

MOLECULAR GENETIC AND IMMUNOLOGICAL STUDIES IN
SYSTEMIC LUPUS ERYTHEMATOSUS.

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Australian National University

by
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STATEMENT

This thesis describes the results of a research project carried out under the supervision of Dr. Sue Serjeantson, at the John Curtin School of Medical Research, Australian National University, from January 1985 to April 1988, during which time I recieved an Australian National University Ph.D scholarship.

The experimental work and data analysis presented in this thesis are my own original work, except when otherwise acknowledged in the text or acknowledgements.

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2/5/88

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ABSTRACT

This thesis examines genetic factors in susceptibility to the autoimmune disease systemic lupus erythematosus (SLE). Candidate genes examined for a possible role in the predisposition to SLE included those encoding the HLA class I and class II antigens, the complement components C4A, C4B and C2, and the T-cell antigen receptor α , β and γ chains. Immunoglobulin allotypes, C3 phenotypes, and lymphocytotoxic activity of SLE sera has also been examined. Genetic profiles were established for approximately 80 Caucasoid SLE patients, for additional patients with other connective tissue disorders and for appropriate controls.

Several earlier studies have shown associations between the HLA class II DR antigens and SLE, but these have often been conflicting. This may be due to the well known difficulty in cellular and serological typing of HLA-D and -DR antigens in SLE patients, and due to possible clinical heterogeneity between SLE patients from different centres. In this study, the technique of DNA-DR typing, which gives unequivocal results, was carried out in parallel with serological HLA-DR typing in SLE and showed a potentially high error rate in HLA-DR serology in lupus. This can be due to incorrect antigen assignment as well as a failure to detect some DR antigens. These errors have the potential to introduce bias into serological studies of HLA-DR in lupus as some DR antigens, such as DR2, are more easily detected than others. Examination of DNA-DR antigen frequencies showed that DR3 alone was increased in Caucasoids with lupus while DR2 was not. Restriction fragment length polymorphism (RFLP) studies of the MHC class II DQ and

DP genes have also been made, but no role for the DQ or DP antigens in SLE was apparent from the RFLP analyses.

Deficiencies of the MHC class III complement components C2 and C4 have also been implicated in the pathogenesis of lupus, but due to linkage disequilibrium with the HLA-B and -DR genes, it has been difficult to distinguish the primary susceptibility locus. In this study, the role of C4 deficiency has been examined in SLE patients from three populations with different MHC gene linkage arrangements. Caucasoid, Japanese and Chinese SLE patients and controls were C4 allotyped and the maximum likelihood method used to calculate gene frequencies. This study showed a significant increase in the C4A null allele (C4A*Q0) frequency in each of the three patient populations when compared with their respective controls, indicating a major role for complete and partial C4A deficiency in the pathogenesis of lupus. Inherited deficiencies of C4B and C2 were not implicated in SLE in this study.

Logistic regression analysis was used to compare the relative contributions of the C4A null gene and DR3 to lupus in Caucasoids, as these two alleles are in significant linkage disequilibrium on the haplotype A1.B8.DR3. This statistical analysis strongly supported the notion that C4A*Q0 is the primary MHC disease promoting locus, with no significant contributions made by HLA-DR3, or C4B*Q0 genes.

However, not all lupus patients have an inherited deficiency of C4A, suggesting genetic heterogeneity in the disease, and some other candidate genes in the pathogenesis of SLE include those encoding the T-cell antigen receptor (TCR). RFLP studies of the TCR- α , - β and - γ genes have been made in Caucasoid SLE patients. No unusual RFLPs or deletions were seen. There was no increase in frequency of any particular DNA

fragments detected with the Tcr- α , - β or - γ chain probes, nor any interaction between TCR RFLPs and HLA class II RFLPs.

Because of the diversity of clinical features in lupus, discriminant analysis has been carried out in an attempt to correlate clinical features of lupus with genetic variables. This revealed a significant decrease in frequency of a DP β /EcoRV RFLP in patients with pleuritis, suggesting a possible role for HLA-DP in some lupus subtypes. Discriminant analysis between SLE and various connective tissue diseases was also performed, and together with inherited partial C4A deficiency, lymphocytotoxic activity against B-cells was an important discriminating variable, suggesting that lymphocytotoxins may be more important pathogenically than previously thought.

This study has shown that SLE is a genetically heterogeneous disease, but one important susceptibility factor, in Chinese and Japanese as well as in Caucasoids, is an inherited partial or complete deficiency of C4A.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION.

Systemic lupus erythematosus (SLE or lupus) is an autoimmune connective tissue disease, the aetiology of which is unknown. Originally, SLE was thought to be a cutaneous disease but in 1872 Kaposi recognized that other organs could be involved and that the disease was therefore systemic in nature. Clearer definition of the systemic features was later provided by Osler, Libman and Sacks, and Klemperer. In 1948, Hargraves discovered the "LE cell" (lupus erythematosus cell) phenomenon which was later shown to represent an antibody to DNP (deoxyribose nucleoprotein). Since then, many other autoantibodies, particularly against nuclear antigens, have been found in the sera of patients with SLE, with the fluorescent antinuclear antibody (ANA) test being used as a routine diagnostic clinical test by the late 1950's. A wide variety of more specific ANAs and other autoantibodies have now been recognized and described, and have contributed to the understanding of the disease (reviewed by Reichlin 1981). The history of lupus has been reviewed in more detail by Graninger et al. (1987).

1.2 EPIDEMIOLOGY.

SLE is a disease primarily affecting females from 15 to 35 years of age, but can occur in all age groups including the newborn and the elderly. SLE has been reported to occur in about one in two thousand Caucasoids (Engleman and Shearn 1983), with a higher incidence observed in several non Caucasoid populations, including American blacks and North American Indians (Steinberg 1985). A study in New Zealand estimated that one in 6780 Caucasoids were affected (Meddings and

Grennan 1980). The differing levels of incidence in Caucasoids may be partly due to ethnic heterogeneity in the Caucasoids studied, or differences in the way that the cases are ascertained. There is no large scale survey of the prevalence of SLE in the Australian community.

1.3 DIAGNOSIS OF SLE.

SLE can vary from a mild cutaneous problem with appropriate serology to a severe, life threatening, multi-organ disease, and can also go through stages of active disease or remission in individuals, with a wide variety of clinical symptoms. In addition, lupus is a disease that can develop very quickly or can take many years to evolve.

The extreme diversity of symptoms seen in lupus, many of which are non-specific, make the diagnosis difficult, particularly when several other connective tissue diseases (CTDs) have the same or similar features. The issue is further complicated by the fact that SLE may well be collection of several diseases rather than one specific disease. This complexity has lead to a number of attempts to develop criteria for the diagnosis of SLE, for use in both epidemiological surveys and in scientific studies, to ensure some uniformity with regard to what is described as a case of lupus.

In 1971, the "Preliminary Criteria for the Classification of Systemic Lupus Erythematosus" (Cohen et al. 1971) were published by the American Rheumatism Association (ARA) which did establish some uniformity in the diagnosis of SLE for clinical and research purposes.

The 1971 criteria have since been updated and revised taking into consideration newly defined immunological markers such as anti-DNA antibodies, antinuclear antibodies, complement levels and complement

TABLE 1.1 1982 REVISED CRITERIA FOR THE CLASSIFICATION OF SLE.

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
5. Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	a) Pleuritis—convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion OR b) Pericarditis—documented by ECG or rub or evidence of pericardial effusion
7. Renal disorder	a) Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed OR b) Cellular casts—may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic disorder	a) Seizures—in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance OR b) Psychosis—in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	a) Hemolytic anemia—with reticulocytosis OR b) Leukopenia—less than 4,000/mm ³ total on 2 or more occasions OR c) Lymphopenia—less than 1,500/mm ³ on 2 or more occasions OR d) Thrombocytopenia—less than 100,000/mm ³ in the absence of offending drugs
10. Immunologic disorder	a) Positive LE cell preparation OR b) Anti-DNA: antibody to native DNA in abnormal titer OR c) Anti-Sm: presence of antibody to Sm nuclear antigen OR d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by <i>Treponema pallidum</i> immobilization or fluorescent treponemal antibody absorption test
11. Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

* The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have systemic lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

(Taken from Tan et al. 1982).

activity (Tan et al. 1982) and these have subsequently been shown to be highly specific for the diagnosis of SLE (Passas et al. 1985). The classification criteria are shown in Table 1.1 (taken from Tan et al. 1982). For a patient to be diagnosed with SLE, he or she must have any four of the criteria, either serially or simultaneously during the course of observation.

It should be mentioned however that these criteria are exclusive, rather than inclusive. The criteria are designed to exclude other autoimmune CTDs, arthritides and certain systemic illnesses (Tan et al. 1982), and therefore may exclude individuals whose diagnosis is almost certainly SLE.

1.4 CLINICAL AND LABORATORY FEATURES OF SLE.

The clinical features of lupus have been described in depth by several authors (Dubois 1976; Ropes 1976; Rothfield 1985) and will only be reviewed briefly here. Table 1.2, (taken from Schur 1983), gives the incidence with which some of clinical features occur in lupus. These frequencies can vary in different studies and may be due to the way in which patients are ascertained.

Most patients with lupus suffer from a number of generalized symptoms seen in many diseases including fatigue, weight loss, fever and a general lack of well being.

Cutaneous disorders of lupus include the diagnostic criteria malar rash, discoid lesions, photosensitivity and mouth and nasal ulcerations. These cutaneous features are very common with 85% of lupus patients affected by some dermatological disorder (Rothfield 1985). Patients are particularly affected by the typical "butterfly " rash across the cheeks

TABLE 1.2 FREQUENCY OF CLINICAL FEATURES IN SLE.

FEATURE	PERCENT
Fever	83
Weight loss	62
Arthritis, arthralgia	90
Skin	74
Butterfly rash	42
Photosensitivity	30
Mucous membrane lesions	12
Alopecia	27
Raynaud's phenomenon	17
Purpura	15
Urticaria	8
Renal	53
Nephrosis	18
Gastrointestinal	38
Pulmonary	47
Pleurisy	45
Effusion	24
Pneumonia	29
Cardiac	46
Pericarditis	27
Murmurs	23
ECG changes	39
Lymphadenopathy	46
Splenomegaly	15
Hepatomegaly	25
Central nervous system	32
Psychosis	15
Convulsions	15

(Taken from Schur 1983).

and nose, and sensitivity to sunlight which can exacerbate the malar rash and erythematous maculopapula eruptions. Alopecia is also fairly common in SLE but is not specific enough to be part of the diagnostic criteria.

Systemic features in SLE involve the joints, kidneys, lungs, heart, central nervous system, gastrointestinal tract, liver and spleen. Joint disease is very common in lupus, ranging from arthralgias and minor tendonitis to joint swelling and arthritis. The arthritis is rarely erosive and deformity is seldom seen in contrast to rheumatoid arthritis (Labowitz and Schumacher 1971).

Renal disease is seen clinically in about 50% of patients, although pathology usually indicates a higher incidence. Renal disorders can vary in severity from mild focal lupus nephritis to acute and ultimately chronic renal failure.

About 50% of SLE patients are affected with lung problems, with pleuritic pain the most common feature. Interstitial lung involvement with acute lupus pneumonitis and diffuse chronic interstitial lung disease can also occur, and lung function abnormalities may be found in asymptomatic patients.

Pericarditis is the most common cardiac disorder in lupus, and is usually acute. Myocardial involvement is uncommon, while vascular disease may be seen at autopsy, but is rarely a problem during life.

Central nervous system manifestations include seizures and psychoses, both of which are diagnostic criteria of SLE. Patients also suffer from headaches (migraine or a more diffuse type), while a small number of patients develop cerebrovascular disease. Peripheral neuropathy has also been described.

Gastrointestinal involvement is not specific enough to be part of

the diagnostic criteria for lupus, and includes anorexia, nausea and vomiting (seen in about 20% of patients), while abdominal pain occurs in about 10% of patients. Hepatomegaly is sometimes seen, but clinical hepatitis is very rare.

Slight to moderate splenomegaly is seen in about 20% of SLE patients, especially those with haemolytic anaemia. Approximately 50% of patients have enlarged lymph nodes at some stage during the course of the disease.

Vascular disease is also seen in lupus, varying from Raynaud's phenomenon (mild to severe), to venous and arterial thrombosis. The thrombosis has been associated with anti-phospholipid antibodies (Harris et al. 1983).

Several haematological abnormalities have been observed in lupus including lymphopenia, leukopenia, thrombocytopenia, and haemolytic anaemia (all part of the diagnostic criteria). Leukopenia occurs in nearly 50% of lupus patients while haemolytic anaemia and thrombocytopenia are seen in about one fifth of patients (Tan et al. 1982). Thrombocytopenia may be due to the presence of anti-platelet antibodies (Maini 1977).

Serology has revealed a number of autoantibodies in SLE including antinuclear antibodies (ANAs, seen in more than 95% of patients), anti-double stranded DNA (anti-dsDNA) antibodies (found in 70% of patients) and anti-Sm antibodies (seen in about 30% of patients), where Sm is an acidic nucleoprotein. Each of these antibodies are diagnostic criteria for SLE, as are the LE cell and a consistently false positive standard test for syphilis.

In addition to the above features, other supporting antibodies include anti-single stranded DNA (anti-ssDNA) antibodies (very common

but not specific), anti-Ro (SS-A) and anti-La (SS-B) antibodies which are also seen in patients with Sjogren's syndrome, and anti-nRNP antibodies which are seen in a high proportion of mixed connective tissue disease (MCTD) patients.

The levels of complement components C3 and C4, and the total haemolytic complement activity are usually decreased during active disease. However, results of complement assays in SLE patients, particularly C4, should be interpreted carefully as inherited C4 null alleles may predispose an individual to SLE.

Cold reactive lymphocytotoxins specific for B- and T-cells have also been observed in SLE sera, which are frequently specific for a patients own lymphocytes (Terasaki et al. 1970). These are of the IgM sub class (Winfield et al. 1975a), and are detectable with the complement dependant microcytotoxicity test. Rheumatoid factor (RF) is another IgM antibody found in the sera of about 20% of SLE patients and is reactive against IgG antibodies (Fong et al. 1985). RF was one of the original criteria for the diagnosis of SLE but has since been omitted as it is not highly specific for SLE.

1.5 ENVIRONMENTAL OR GENETIC?

Both environmental and genetic factors have been implicated in the pathogenesis of SLE. Studies have shown that the age of onset of SLE in sibling pairs occurs closer in the calendar year than in the actual age of the siblings (Arnett and Schulman 1976; Kaplan et al. 1984) which would indicate an environmental agent. Further support comes from finding anti-nucleic acid antibodies (De Horatius et al. 1975) and anti-lymphocyte antibodies (De Horatius and Messner 1975) increased in

frequency in the sera of close household contacts of patients with SLE. Laboratory workers who handle SLE blood also have a higher incidence of lymphocytotoxic antibodies (LCAs) compared with the general population (De Horatius et al. 1979).

On the other hand, close blood relatives of SLE patients, with little patient contact, have an increased incidence of LCAs (De Horatius and Messner 1975) and a study by Elkon et al. (1983) has shown an increase in circulating immune complex levels in first degree relatives of SLE patients, both with and without close household contact, which would tend to support a genetic basis. In addition, there is a higher concordance rate of disease in identical twins (57%) compared to dizygotic twin pairs and sibs (Block et al. 1975) with disease expression very similar between identical twins. It has been observed that approximately 10% of SLE patients have a first or second degree relative with SLE (Arnett et al. 1984) which is suggestive of genetic involvement.

The most convincing evidence for the role of genetic factors in the pathogenesis of SLE is provided by the association of major histocompatibility complex (MHC) genes with the disease, as reviewed later in this chapter and Chapter 3. Further evidence for genetic involvement is provided by animal models of SLE.

1.6 ANIMAL MODELS OF SLE.

To further understand the genetics of SLE, studies have been made of animals which have lupus like diseases such as certain mouse and dog strains. The most studied mouse strain has been the New Zealand Black x New Zealand White F1 [(NZB x NZW)F1] mouse which develops a lupus like

disease very similar to that seen in humans. The parental NZW mouse is phenotypically normal, and any autoimmune symptoms are rarely seen before 18 months of age. The NZB mouse does have some autoimmune manifestations such as haemolytic anaemia and occasionally anti-ssDNA or anti-dsDNA or anti-histone antibodies. Renal disease rarely occurs when the NZB mice are less than one year old and is very mild. However, in the (NZB x NZW)F1 hybrids, anti-dsDNA and anti-histone antibodies are present, and the mice suffer from fatal immune complex glomerulonephritis. The female F1 mice have an average life span of only eight months. Some new strains of mice with lupus like diseases are now available for study such as the MRL mouse and BXSB mouse. However, none of the mice have the same H-2 type (the mouse equivalent of the human MHC genes), nor the same immunoglobulin heavy chain type. Work on the T cell receptor (TCR) in these mice strains indicates that the NZW mice do have differences in their TCR genes, but whether these have an effect on the pathogenesis of the lupus like disease in the mice is not clear. Different accelerating factors are also seen in the mice. For example, hormones play a role in the (NZB x NZW)F1 mice with female mice dying much sooner than male mice and the lymphoproliferative (lpr) gene in MRL mice reduces life expectancy three-fold. How these accelerating factors work is unknown. The murine models suggest that there are several mechanisms or abnormalities which can lead to lupus; the mice lupus models are reviewed by Theofilopoulos et al. (1986).

1.7 CANDIDATE GENES FOR SLE IN HUMANS.

Several genes are candidates for causing the immunological abnormalities seen in lupus such as the genes of the major

histocompatibility complex and the T-cell receptor (TCR) genes. The molecular organization, structure and function of the MHC and T-cell receptor genes have been the subject of intense investigation and are reviewed here in some detail.

1.8 THE MAJOR HISTOCOMPATIBILITY COMPLEX.

The MHC is found on the short arm of chromosome 6, at the 6p21.3 band, and encodes some of the most polymorphic human genes. The MHC is comprised of three classes of genes, (Figure 1.1), which encode the class I HLA-A, -B, and -C antigens found on the surface of the majority of nucleated cells, the class II HLA-D antigens found primarily on the surface of B-lymphocytes, and the class III serum complement components C2, C4 and BF and also the 21-hydroxylase enzyme. Recently, the tumor necrosis factor (TNF) α and β chain genes have been localized between the class I and III genes.

1.8.1 THE HLA-A, -B, AND -C ANTIGENS.

The class I antigens, (the classic transplantation antigens), consist of a polymorphic transmembrane glycoprotein about 45kDa in size encoded in the MHC, and a noncovalently associated 12kDa β 2 microglobulin (Strominger et al. 1977) which is encoded by a gene on chromosome 15 (Goodfellow et al. 1975). The heavy chain has three external domains, a transmembrane domain and a cytoplasmic domain. The β 2 microglobulin is associated with the highly conserved third external domain next to the cell membrane, while the first two extracellular domains are the most polymorphic (Yokoyama and Nathenson 1983).

Polymorphism in the class I antigens is evident by serological, cellular and biochemical techniques with the Ninth International Histocompatibility Workshop recognizing 23 HLA-A, 47 HLA-B and eight HLA-C antigens, which were detected serologically (Bodmer and Bodmer 1984). The class I antigens are important in the discrimination between self and nonself during an immune response, as they are found on the majority of cells, and therefore play an important role in transplantation. In addition, T-cytotoxic cells recognize antigen in association with the MHC class I molecules during an immune response.

1.8.2 THE HLA-D ANTIGENS.

The class II HLA-D antigens are polymorphic heterodimers comprised of a heavy α chain about 33 kDa in size and a light β chain (28 kDa) (Snary et al. 1977; Springer et al. 1977). Each polypeptide chain has two extracellular domains, the first showing the most polymorphism, a transmembrane domain and a cytoplasmic domain (Kaufman and Strominger 1982). The first external domains of the α and β chains probably combine together to bind antigen. Three HLA-D antigens have so far been detected, the DR, DQ and DP antigens, with the possibility of a fourth indicated from molecular genetic (Trowsdale et al. 1985) and biochemical studies (Carra and Acolli 1987). The Ninth International Histocompatibility Workshop recognized 14 DR, three DQ and six DP serologically distinct antigens (Bodmer and Bodmer 1984). During an immune response, B-cells, macrophages and other antigen presenting cells (which carry the class II antigens on their cell surface), process foreign antigen and present it on the cell surface in association with the class II proteins. T-cells, with their T-cell receptor which

recognizes foreign antigen in association with the class II protein, are then able to interact and become activated and function as T-helper cells, stimulating B-cell involvement and antibody production.

1.8.3 GENETIC ORGANIZATION OF THE MHC CLASS II GENES.

The organization of the genes in the MHC is shown in Figure 1.1. The MHC covers an area of about 2.5cM and the order of the genes was established initially with family studies of people with HLA recombinant haplotypes, both serologically and by recombinant DNA techniques. More recently, the new method of pulsed field gel electrophoresis (PFGE) and the use of overlapping cosmid clones (Dunham et al. 1987; Carroll et al. 1987) has proved very useful as larger areas of DNA can be covered.

The class II region contains four sets of genes, the DR, DQ, DP, and DO/DZ genes. By linkage of cosmid clones (Korman et al. 1985) and southern blotting (Wake et al. 1982a; Bohme et al. 1983), the DR locus has been shown to consist of one α gene and a variable number of β chain genes. The number of β chain genes correlates with serological DR types (Bohme et al. 1985). For example, DRw8 has one β gene while DR4 has at least 3 β genes (Bohme et al. 1985). The $\beta 2$ gene appears to be a pseudogene in some DR types and it is possible that it has played a role in gene conversion (Larhammar et al. 1985; Rollini et al. 1985). The DR α gene shows restricted polymorphism (Larhammar et al. 1982; Stetler et al. 1982; Wake et al. 1982a) but the β genes are highly polymorphic (Bohme et al. 1983; Wake et al. 1982b).

There are two pairs of genes in the DQ region, known as DQ α and DQ β , and DX α and DX β , which have not been linked with cosmid cloning (Okada et al. 1985a; Korman et al. 1985). The DQ RFLPs show strong linkage disequilibrium with DR types unlike those at the DX locus (Auffray et al. 1984; Trowsdale et al. 1983; Spielman et al. 1984), and with supporting evidence from deletion mutant cell lines it has been suggested that the DX genes are centromeric to the DR and DQ genes, (Auffray et al. 1983a), and that there is a hot spot for recombination between the DX and DR/DQ genes (Trowsdale et al. 1985). The DX gene pair do not appear to be expressed as no mRNA or protein has yet been detected, but from sequencing, the genes do not appear to be pseudogenes (Auffray et al. 1984; Okada et al. 1985a). The DQ α and DQ β genes show variability in the splicing points at their 3' ends which can result in deletion of part of the untranslated region (Schenning et al. 1984). The DX gene pair show limited polymorphism but both the DQ α and DQ β genes have extensive polymorphism when examined for RFLPs (Auffray et al. 1983b; Spielman et al. 1984; Cohen et al. 1984). The variability in the DQ α gene has been shown to be clustered to amino acid residues in the first external domain (Chang et al. 1983; Trowsdale et al. 1985).

The DP region covers about 100kb of DNA (Okada et al. 1985b) and has been extensively characterized (Roux-Dosseto et al. 1983; Gorski et al. 1984; Trowsdale et al. 1985). Two pairs of genes have been found in this region, namely DP α 1 and DP β 1, and DP α 2 and DP β 2 and these have been linked by cosmid cloning. Sequencing suggests that the α 2 and β 2 gene pair are pseudogenes due to frame shift mutations and defective slicing sites (Kappes et al. 1984; Serenius et al. 1984). Sequencing of the DP genes (Kelly and Trowsdale 1985) and the DP protein (Hurley et al. 1982) indicates that α 1 and β 1 encode the DP antigen. Different splicing

patterns have been observed as well as variation in the site for the poly A tail (Kelly and Trowsdale 1985). The DP genes were originally thought to show little polymorphism (Spielman et al. 1984), but it is now evident that they are highly polymorphic, with genetic variation clustered in regions of the second exon (Bugawan et al. 1987).

The fourth set of genes in the HLA-D region are the newly identified DO β and DO α /DZ α genes. The DZ α gene was originally identified by Spielman et al. (1984) and was later shown to be identical to the DO α gene which was isolated by Inoko et al. (1985). DO appears to lie between DQ and DP from PFGE and cosmid cloning studies (Hardy et al. 1986; Erlich et al. 1986; Amar et al. 1987). DO β and DO α /DZ α may not be a gene pair as PFGE indicates that they lie about 100kb apart in the MHC and DO α /DZ α is inducible by γ interferon while DO β is not. From their sequences, both could be functional genes. Northern blots have shown a low level of expression of the DO genes in some B-cell lines, but a very large mRNA was detected (Trowsdale et al. 1985). No RFLPs have yet been found (Trowsdale et al. 1985).

1.9 ASSOCIATION OF THE CLASS I AND II GENES WITH DISEASE.

The class I and II genes have shown associations with several diseases which are characterized by abnormalities in the immune system such as insulin dependent diabetes mellitus, rheumatoid arthritis, and multiple sclerosis. These genetic associations may be directly attributable to defects in the class I and II genes such as deletions, splicing errors, or deviation in the exon sequences from normal. On the other hand, as a 100% association with the HLA antigens is not seen in these diseases, the HLA genes may be markers for the disease

susceptibility gene (a non HLA gene) which could be carried on the same haplotype. A high level of linkage disequilibrium is seen in the MHC and may be a consequence of selective pressure for certain beneficial genes, for example during a disease epidemic. However, this linkage disequilibrium may have resulted in some less beneficial genes (with regard to autoimmunity) being carried on the haplotype, a feature which has been called "hitch hiking" (Bodmer and Bodmer 1978). There is still an area of the MHC, between the class I and III genes, where new genes could be located, or a disease susceptibility gene could lie outside the MHC. A third explanation for disease associations with genes in the MHC is that certain MHC types are necessary, but not sufficient, for the development of disease, with other non-MHC genes and environmental factors contributing to disease development in autoimmune disorders.

1.9.1 ASSOCIATION OF THE CLASS I AND II MHC GENES WITH SLE.

SLE was originally reported to be associated with HLA-B5 and HLA-B8 in several populations, but it was later shown that the class II antigens DR2 and DR3 were also increased in frequency in SLE (reviewed by Tiwari and Terasaki 1985). However, because of the linkage disequilibrium seen in the MHC, as observed between B8 and DR3 for example, it was not possible in studies of small numbers of patients to identify which locus conferred the greatest risk for SLE. Further, these HLA genes could be part of a haplotype which carries a disease susceptibility gene, such as defective genes at the class III locus.

1.10 THE CLASS III GENES IN THE MHC.

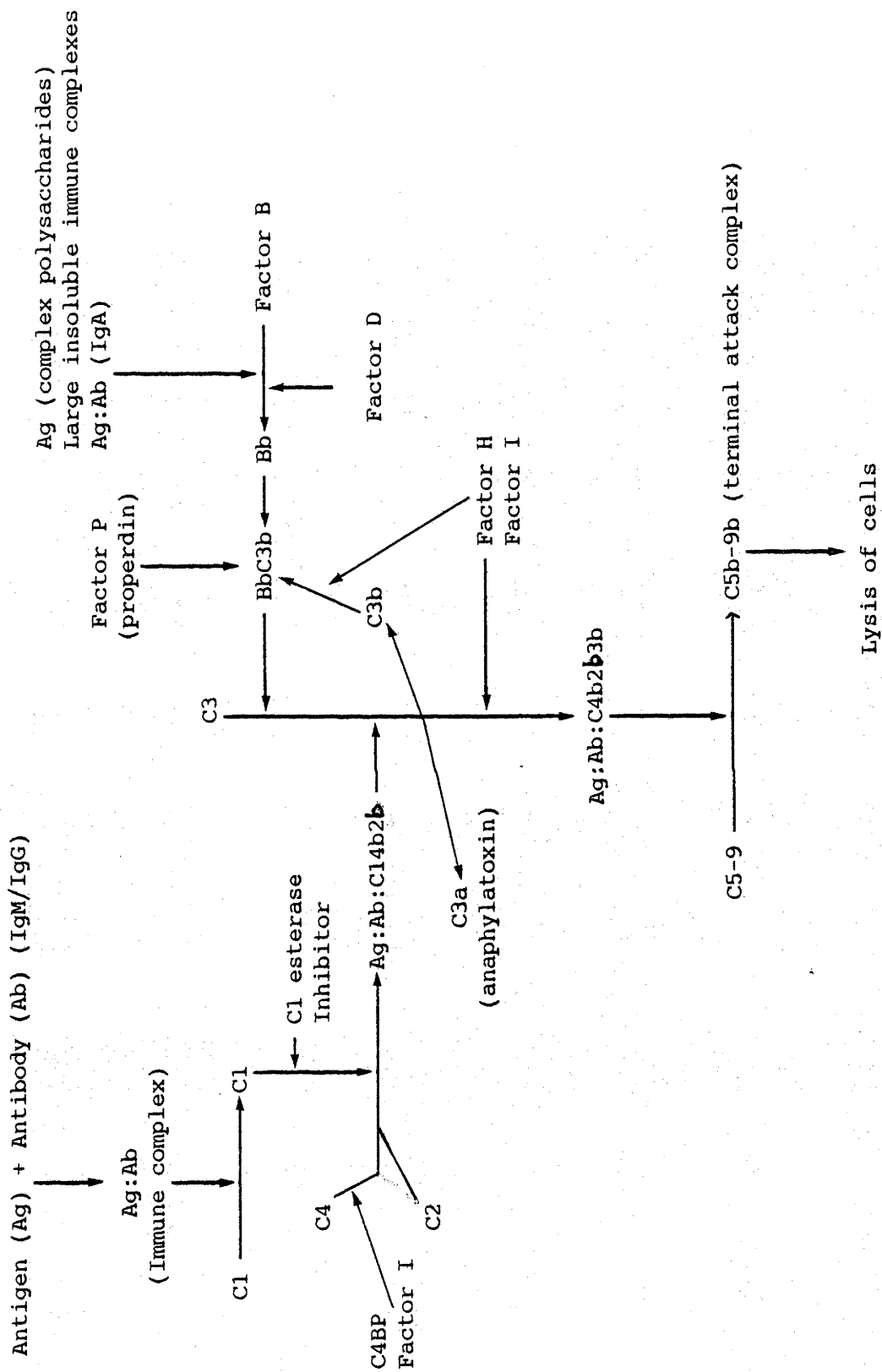
The early acting complement components C2, C4, and BF are encoded in the class MHC III region along with the genes for 21-hydroxylase (Figure 1.1). Mapped with overlapping cosmid clones (Carroll et al. 1984) the class III genes lie between the class I and HLA-D genes with C2 and BF only 421bp apart, while the C4 genes are about 30kb away from BF. The C4A and C4B genes lie about 10kb apart from each other while the 21-OH genes are located close to the 3' ends of the C4 genes (Carroll et al. 1985; White et al. 1985). PFGE has established the gene order as HLA-B, C2, BF, C4A, 21-OHA, C4B, 21-OHB, HLA-DR (Dunham et al. 1987; Carroll et al. 1987).

1.10.1 THE COMPLEMENT SYSTEM.

The complement system is comprised of about 18 plasma proteins which are involved in a series of sequential reactions and which show a wide variety of immunological activities including solubilization of immune complexes, viral neutralization, lysis of foreign cells, opsonization, and stimulation of the inflammatory response. There are two pathways in the complement system - the classical, and the alternative which join together in the terminal attack cascade (Figure 1.2).

In the classical pathway, complement component C1 is activated by antigen:antibody complexes (Ag:Ab), particularly those with IgG or IgM antibodies, resulting in the activation of C1. The C1s subunit of C1 in turn activates complement component C4, cleaving 10kDa, (C4a, released), from the amino terminal of the α chain of C4 and leaving C4b which binds

FIGURE 1.2 THE COMPLEMENT CASCADE.



to the Ag:Ab:C1 complex. In the presence of magnesium ions, C2 binds to C4b and is cleaved by C1s to C2a (released) and C2b. C4b2b is a C3 convertase enzyme, cleaving C3 to C3a and C3b through a proteolytic site on C2b. C3a, an anaphylatoxin, is released into the circulation and C3b binds to the Ag:Ab:C14b2b complex and cleaves complement component C5 to its active form, resulting in the activation of the terminal attack cascade and the formation of C5b-9b, (the terminal attack complex), which is extremely hydrophobic and inserts into the lipid bilayer of cells and causes lysis.

In the alternative pathway, factor B (BF) is activated by antigens particularly those carrying complex polysaccharides on their surface such as bacteria, as well as large insoluble immune complexes and IgA carrying immune complexes. Factor D cleaves factor B forming Ba (released) and Bb which binds to C3b. Properdin stabilizes this C3 convertase and the terminal attack pathway is activated. A positive feedback loop, controlled by Factors I and H, is set up as C3 is part of its own C3 convertase. In the classical pathway, C1 is controlled by C1 esterase inhibitor and C4 is inactivated by C4 binding protein and factor I which cleaves C4b to C4c and C4d (for general references on the complement system see Dias da Silva 1986; Schur 1985).

Both pathways act together to clear immune complexes from the body, the alternative pathway being essential for the solubilization of immune complexes while the classical pathway is essential for the prevention of precipitation of immune complexes, (Webb and Whaley 1986; Schifferli 1986a). To facilitate the clearance of immune complexes from the circulation, C3b and C4b bind to a receptor, CR1, which is found in large numbers on erythrocytes so that immune complexes can be transported to the liver where the fixed macrophage system removes them.

This prevents interaction of immune complexes with endothelium and reduces local inflammation and tissue damage from the activation of complement. C3b also plays a role in opsonization, coating immune complexes which facilitates binding to macrophages which carry a C3b receptor (Bianco et al. 1975).

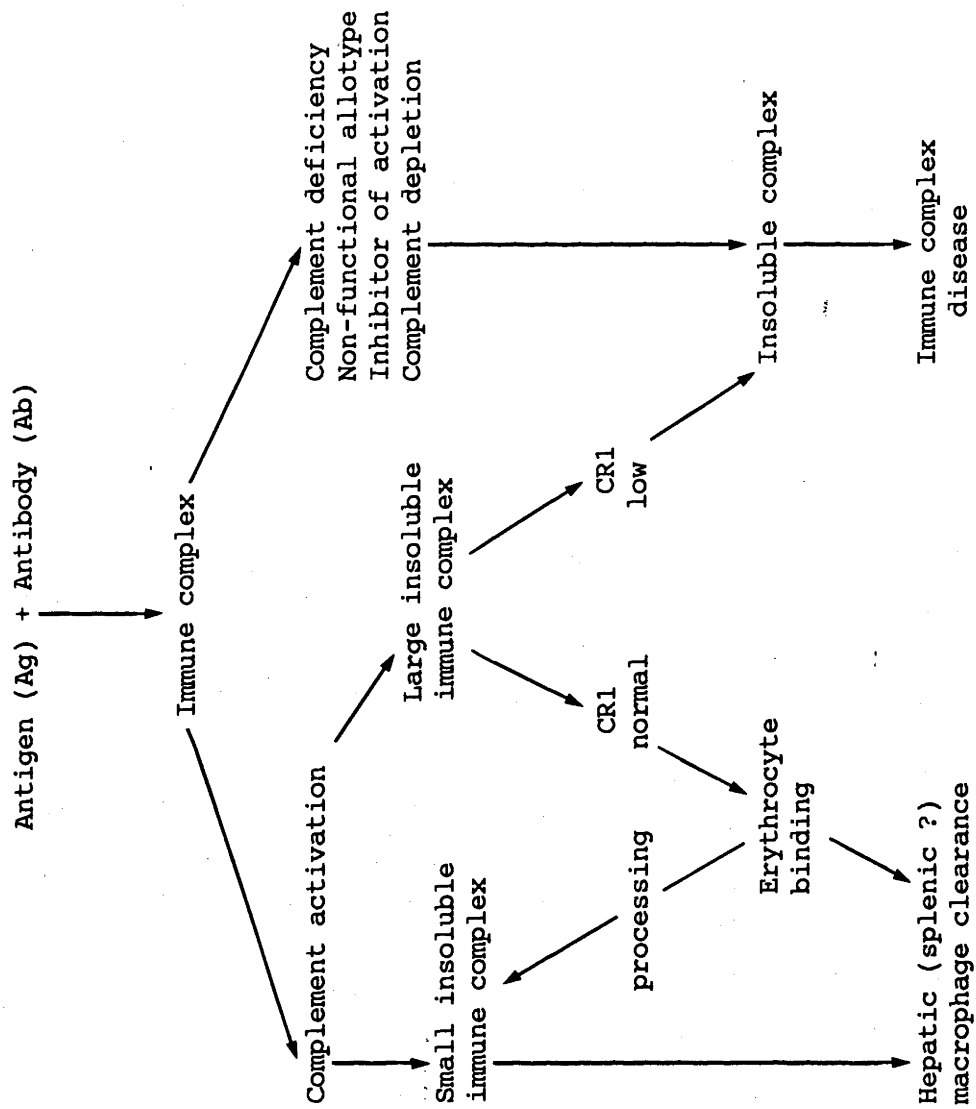
1.10.2 COMPLEMENT DEFICIENCIES.

Deficiencies of most of the complement proteins are seen in the general population with approximately equal numbers of males and females affected. C2 deficiency is the most common with about 1% of Caucasoids heterozygous for C2 deficiency (Glass et al. 1976). Individuals with complete deficiencies of one of the early complement components C1, C2, or C4 often present with a lupus-like disease but usually lack the characteristic LE cell, antinuclear antibodies or Ig and C3 deposits in the skin, which are seen in lupus. Deficiencies of components C5 to C8 result in an increased susceptibility to bacterial infections, in particular to the bacterium Neisseria, while complete lack of C3 causes predisposition to extremely severe, recurrent diseases usually caused by bacterial infection. Complement component C9 deficiency has been seen in many people but does not appear to predispose individuals to any particular disease. Factor P (properdin) deficiency results in high susceptibility to meningococcal meningitis. Complement deficiencies are reviewed by Schur (1986) and Agnello (1986). Complete deficiency of factor B has not been reported indicating that it may be lethal, but silent alleles have been reported in some families (Suciu-Foca et al. 1980; Tokunaga et al. 1984; Bertrams and Mauff, 1985).

The early complement components C2, C4, and BF are encoded by genes in the class III region of the MHC, and are therefore important in disease studies because of the association of the MHC class I and II genes with diseases such as IDDM (Svejgard 1980) and because of the linkage disequilibrium between genes in the MHC. Due to the linkage disequilibrium it is often difficult to pinpoint the locus conferring the greatest risk in diseases such as SLE where associations are sometimes weak. However, in the case of SLE where deficiencies of complement components C1q, C1r, and C1s are associated with a lupus-like disease (Schur 1986; Agnello 1986) and are not encoded by genes in the MHC and where acquired deficiencies of C4 and C2 (eg. from C1 esterase inhibitor deficiency) (Frank et al. 1976) also result in a lupus-like disease, it would appear that low levels of complement could predispose an individual to SLE. This could happen in several ways. For example, a complement deficient individual may not be able to eliminate a pathogen from the body fast enough so that it causes a chronic illness or else the subject may not be able to process immune complexes and these could be deposited causing the tissue damage seen in lupus.

Figure 1.3 taken from Webb and Whaley (1986) shows possible pathways to immune complex disease. An increase in circulating immune complexes could result from inherited complement deficiencies, non functional complement allotypes, inhibitors of complement activation, complement depletion or low levels of CR1 (the C3b/C4b receptor), therefore increasing the chances of the formation of insoluble immune complexes and precipitation and the development of immune complex disease.

FIGURE 1.3 PATHWAYS TO IMMUNE COMPLEX DISEASE.



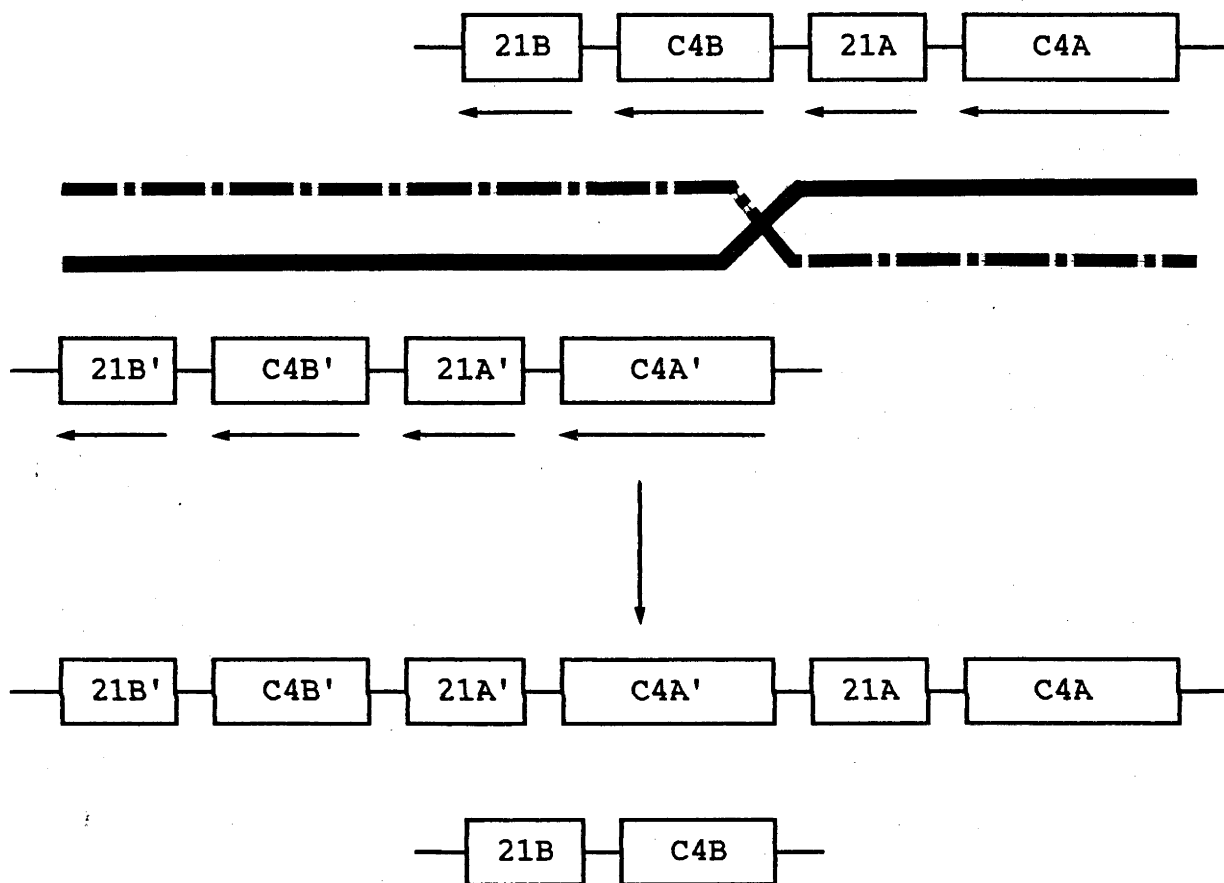
(Taken from Webb and Whaley 1986).

1.10.3 COMPLEMENT COMPONENT C4.

Complement component C4 is encoded by two genes in the MHC, C4A and C4B (O'Neill et al. 1978), which arose through gene duplication (White et al. 1985) and which show considerable polymorphism with at least 13 C4A and 21 C4B alleles detectable by agarose gel electrophoresis of desialized C4 followed by immunofixation (Mauff et al. 1983), making C4 one of the most polymorphic serum proteins in Caucasoids. More polymorphism is evident at the DNA level with a few reported RFLPs already subdividing some of the known C4 alleles (Whitehead et al. 1984). Only one RFLP specific for a C4 allele, (C4A*6), has been reported (Palsdottir et al. 1983). In addition to the variation in C4 generated by the large number of alleles at each locus, there can be duplication of C4A or C4B loci on some haplotypes (Bruun-Peterson et al. 1982; Raum et al. 1984; Uring-Lambert et al. 1984), and null alleles of both C4A and C4B have been observed.

C4 is synthesized in a proenzyme form, about 200 kDa in size and 1722 amino acids in length, which is cleaved to produce a protein comprised of three polypeptide chains α (95 kDa), β (75 kDa), and γ (30 kDa) linked by disulphide bonds (Schreiber and Muller-Eberhard 1974; Reid and Porter 1981). The α chain of C4A is about 96 kDa in size while that of C4B is 94 kDa, however they have been shown to be identical in length suggesting that conformational changes are responsible for the observed size differences (Roos et al. 1982). Sequencing has shown that there are only 15 nucleotides which are different between the C4A and C4B proteins (Belt et al. 1985), twelve of which are located close to the internal thiolester bond and are responsible for the different alleles observed and their functional heterogeneity. These base changes

FIGURE 1.4 A POSSIBLE MECHANISM FOR DELETION OF THE C4A GENE.



result in amino acid substitutions, six are believed to be isotypic and four allotypic. C4A gene products show less haemolytic activity than those of the C4B gene, with C4A*6 on certain haplotypes showing the least haemolytic activity of all (O'Neill et al. 1980; Teisberg et al. 1980). Dodds et al. (1985) have shown that the C5 convertase formed with C4A*6 is less active which explains the lower haemolytic activity of C4A*6.

Homozygous C4A deficiency is not common in the general population, estimated to be present in about 1% of individuals while homozygous C4B deficiency is more prevalent at the 5% level (O'Neill et al. 1978). Complete C4 deficiency is rare and is usually associated with SLE (Agnello 1986). In about 50% of cases, C4 null alleles are believed to have arisen through deletions (Schneider et al. 1986), which are probably due to unequal crossing over, resulting in one chromosome having a deletion in a C4 gene (Raum et al. 1984; Schneider et al. 1986) (Figure 1.4), but in other cases transcriptional or translational defects are believed responsible. It has also been suggested that a C4 gene could be converted to code for the product at the alternate locus, therefore only C4A or C4B alleles would be observed (Palsdottir et al. 1987). In one family, C4 deficiency has been associated with a gene which is not linked to the MHC (Muir et al. 1984). The deficiency was incomplete and appeared to be autosomal dominant.

1.10.4 COMPLEMENT COMPONENT C2.

Complement component C2 is encoded by a single gene in the MHC which shows 39% amino acid sequence homology (Campbell 1987) and similar organization to that of Factor B, (the equivalent of C2 in the

alternative pathway), indicating they probably arose through gene duplication. The C2 gene encodes a 102 kDa glycoprotein (Kerr and Porter 1978), which is 732 amino acids in length. C2 is a serine protease but shares an uncommon feature with BF in being much longer than the majority of serine proteases. C2 does not show such extensive polymorphism as C4 with 4 alleles detectable with isoelectric focussing followed by a haemolytic overlay, but like C4, greater variation is seen at the DNA level (Bentley et al. 1985; Cross et al. 1985).

Southern blot analysis of the C2 gene has shown no major rearrangements or deletions in individuals who are C2 deficient (Cole et al. 1985). The lack of detectable mRNA in these people led Cole and his colleagues to propose that a transcriptional or pretranslational defect leads to C2 deficiency. C2 deficiency is strongly associated with the MHC haplotype A25.B18.BF*S.C4A*4.C4B*2.DR2(Dw2) with about 67% of C2 deficient individuals carrying this extended haplotype (Hauptmann et al. 1982). This makes it difficult to say whether the complement deficiency or another gene (or genes) on the haplotype are important in the pathogenesis of SLE.

1.11 STRUCTURE AND FUNCTION OF THE T-CELL RECEPTOR.

Another group of genes which could be involved in SLE are those encoding the T-cell antigen receptor. The TCR recognizes foreign antigen in association with the HLA antigens and a defect in the TCR could conceivably lead to autoimmunity.

1.11.1 THE $\alpha\beta$ RECEPTOR.

The T-cell antigen receptor (TCR) is a polymorphic heterodimer responsible in part for discrimination between self and nonself (Yague et al. 1985), conferring clonal variability in T-cell recognition of antigen/MHC complexes (Dembic et al. 1986). The TCR has a protein structure similar to other members of the immunoglobulin superfamily with the characteristic immunoglobulin homology unit and both constant and variable domains (Hunkarpillar and Hood, 1986). There are two types of T-cell receptor, the first is comprised of an acidic α and a basic β polypeptide chain linked with a disulphide bond. The α chain is about 45 kDa in size while the β chain is about 40 kDa (Allison et al. 1982; Haskins et al. 1983; Meuer et al. 1983a; Meuer et al. 1983b). The $\alpha\beta$ receptor is found on the surface of the majority of T-cells and is MHC restricted in activity - that is, it requires compatible or self MHC protein to react. The $\alpha\beta$ receptor on the T-helper subset of lymphocytes recognizes antigen in association with an MHC class II protein presented on the surface of antigen presenting cells such as macrophages (Epplen 1987). Another T-helper cell surface protein, CD4, is also involved in this recognition process, possibly increasing the affinity between the T-cell and the antigen presenting cell by binding to the MHC class II molecule (Epplen et al. 1987). A third T-cell protein, CD3, comprised of three polypeptide chains γ , δ and ϵ , (Borst et al. 1983), is noncovalently associated with the T-cell receptor and is thought to transmit a signal into the cell which triggers T-cell activation (Weiss and Stobo 1984; Oettgen et al. 1985; Samelson et al. 1985). T-cytotoxic cells also carry the $\alpha\beta$ receptor but ^{usually} recognize antigen in association with the MHC class I molecule (Epplen 1987). The T-cytotoxic cell

protein, CD8, is thought to be involved in the recognition of the MHC protein and CD3 is believed to transmit an activation signal across the cell membrane.

1.11.2 THE $\gamma\delta$ RECEPTOR.

The second type of T-cell receptor is comprised of a γ and a proposed δ polypeptide chain (Brenner et al. 1986; Moingeon et al. 1986) with the γ polypeptide chain about 55kDa in size and the putative δ chain about 40kDa (Brenner et al. 1986; Littman et al. 1987). This receptor is expressed early in T-cell maturation which takes place in the thymus. In adults, the $\gamma\delta$ receptor is found mainly on the least mature T-cells in the thymus, (Fowlkes et al. 1985), and on the $CD3^+CD4^-CD8^-$ subset of peripheral T-cells, (about 2% of T-cells), (Brenner et al. 1986; Borst et al. 1987), with both a disulphide and non-disulphide linked form of the $\gamma\delta$ receptor having been observed (Brenner et al. 1987; Borst et al. 1987). Unlike the $\alpha\beta$ receptor carrying cells, T-cells with the $\gamma\delta$ receptor are not MHC restricted in their cytotoxic activity (Borst et al. 1987; Brenner et al. 1987). This $\gamma\delta$ receptor is associated with the CD3 protein complex which has been shown to be involved in signal transduction and activation of Tcr- $\gamma\delta$ lymphocytes (Weiss et al. 1986; Krangel et al. 1987).

1.11.3 T-CELL RECEPTOR GENES.

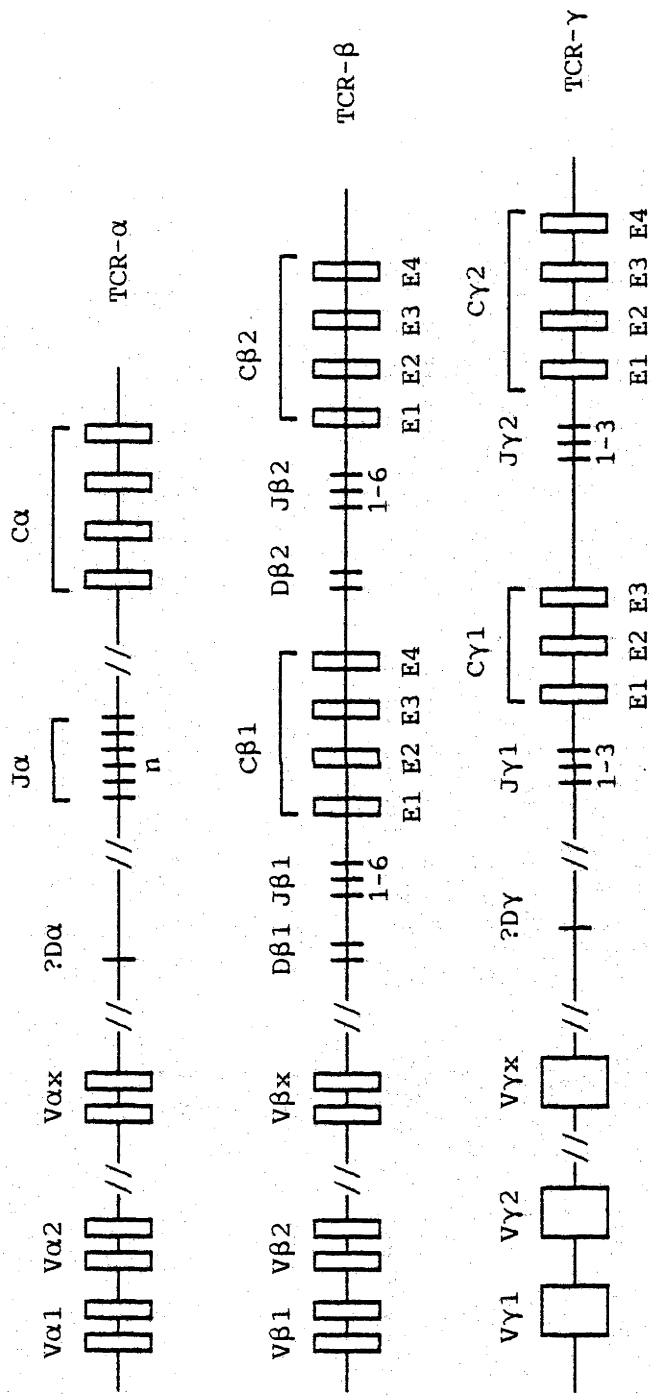
The genes for the TCR- α , TCR- β and TCR- γ polypeptide chains have been localized and characterized, the β gene maps to chromosome 7 (Caccia et al. 1984) at 7q32-35 (Lebeau et al. 1987; Morton et al.

1985), while the γ gene is believed to be at 7p15 (Murre et al. 1985). The TCR- α gene is found on chromosome 14 at 14q11-12 (Caccia et al. 1985; Collins et al. 1985). cDNA clones believed to encode the TCR- δ protein have recently been isolated (Hata et al. 1987; Band et al. 1987), and the gene is believed to lie just upstream of the TCR- α gene cluster (Takahara et al. 1987).

1.11.3.1 TCR- β GENE ORGANIZATION.

There is a high degree of similarity between the immunoglobulin genes and those encoding the T-cell receptor. Like the immunoglobulin genes, the TCR genes are encoded by variable (V), joining (J), and constant (C) gene segments, and in the case of TCR- β there is also a diversity (D) gene region (Figure 1.5). The TCR- β gene was the first isolated and therefore is the best characterized TCR gene. Of all the TCR genes it is the one most like that of the immunoglobulins. There are between 60 and 100 TCR- β variable genes (Concannon et al. 1986a; Kimura et al. 1986) with each variable region gene comprised of two exons, one for the leader sequence and one for the protein variable domain (Siu et al. 1984). The 3' ends of the V region genes are very diverse (Concannon et al. 1986b) and it has been shown that some V region genes are utilized more often than others (Behlke et al. 1985; Sim and Augustin, 1985). Between 25-30% of the variable genes are thought to be pseudogenes, with maybe 5% or less V region genes actually used (Concannon et al. 1986a). The diversity and joining region genes are organized into two tandemly linked clusters each associated with a single constant region gene, with this organization probably arising through gene duplication (Toyonaga et al. 1985). The D-J-C region

FIGURE 1.5 GENETIC MAP OF THE α , β AND γ T-CELL RECEPTOR GENES IN MAN.



covers about 24kb of DNA with six J region genes in each cluster and the two C regions about 10kb apart (Toyonaga et al. 1985; Mak et al. 1986). The two C regions are composed of four exons each, and show similar sequence and genomic organization, the first two exons encode the extracellular domains, the third the transmembrane domain and the fourth the cytoplasmic carboxy terminal domain and the 3' untranslated region. The 95 bp 3' to the first exon have been shown to be highly conserved among C region genes, but the other intron sequences are not (Toyonaga et al. 1985). Rearrangements between the V, D, and J gene segments occur with the use of recombination signals similar to those seen in the immunoglobulin genes (Early et al. 1980; Tonegawa 1983). These are immediately adjacent to the V, D, and J coding sequences and comprise a highly conserved heptamer followed by a variable spacer and then an AT rich nanomer (Toyonaga et al. 1985; Clark et al. 1984; Siu et al. 1984). The spacer in between the heptamer and the nanomer is either 12 or 23 bp in length, the 12 bp spacer is found 5' to the D- β and J- β genes and the 23 bp spacers are found 3' to the V- β and D- β genes. Early and his colleagues showed that a short spacer is always involved in recombination with a long spacer in the immunoglobulin genes (Early et al. 1980). Therefore, the arrangement of spacers seen in the TCR genes allows optional use of the D and J gene segments.

1.11.3.2 TCR- α GENE ORGANIZATION.

Unlike the TCR- β gene, the TCR- α gene does not appear to contain diversity gene segments but does have a large number of V genes, estimated to be from 50 (Yoshikai et al. 1986) to more than 100 (Klein et al. 1987). The V region genes can be divided into closely related

cross-hybridizing families (Yoshikai et al. 1986). Like the TCR- β V genes, the TCR- α V genes are composed of two exons, the first a signal peptide and the second encoding the variable protein domain (Yoshikai et al. 1985). It has been observed that the coding sequences of the joining regions are several codons longer than those of TCR- β and the immunoglobulins and there are many more of them, (Yoshikai et al. 1985) covering an area of about 70kb of DNA and separated by about 1kb. The first J region gene is found about 4kb upstream of the single constant TCR- α gene. Recombination signals like those seen in the TCR- β gene are located next to the variable and joining gene segments (Yoshikai et al. 1985; Baer et al. 1986). The C region genes are comprised of four exons, the first two encoding the extracellular domains, the third the transmembrane and cytoplasmic tail domains and the fourth the 3' untranslated region. The unusual feature of the poly A tail being almost entirely encoded by one exon is also seen in the MHC genes (Baer et al. 1986; Yoshikai et al. 1985).

1.11.3.3 TCR- γ GENE ORGANIZATION.

The TCR- γ gene locus contains at least 9 variable regions covering an area of about 54 kb, which can be divided into two groups on their DNA homology, one group with 8 members, the other with one (LeFranc et al. 1986a). Of the former group, 4 of the 8 have been shown to be pseudogenes (LeFranc et al. 1986a). Recombination signals like those in the immunoglobulin and other TCR genes have been observed 3' to the variable gene segments and 5' to joining segments (LeFranc et al. 1986a). There are two constant genes, about 16kb apart, each associated with several joining regions (LeFranc and Rabbitts 1985). The Cy1 gene

has three exons but CY2 has 4 exons as the second exon has been duplicated and both copies have lost the codon for the cysteine residue which is thought to be involved in forming the disulphide bond between the γ and δ polypeptide chains (LeFranc et al. 1986b; Pelicci et al. 1987). This explains the two forms of the $\gamma\delta$ TCR which have been seen, with use of the Cy1 gene resulting in the disulphide linked heterodimer and use of the Cy2 gene in the non-disulphide linked form (Krangel et al. 1987; Littman et al. 1987).

1.11.4 DIVERSITY OF THE T-CELL RECEPTOR.

Diversity in the T-cell receptor is generated by flexibility in the joining position of the V, (D), J and C regions when they are rearranged as well as the many possible combinations of gene segments. In addition, random nucleotides can be added during joining of the V, J, (and D) gene segments, (N-region diversity), a phenomenon also seen in the rearrangements of the heavy chain immunoglobulin genes (Alt and Baltimore 1982). However, unlike the immunoglobulin genes which can also generate variability by somatic hypermutation, TCR genes do not use somatic mutation, probably because T cells are first selected for nonreactivity against self antigens before leaving the thymus, and further somatic mutation after this could result in self reactive clones.

1.12 AIMS OF THESIS.

No definite susceptibility gene has yet been found in SLE despite some intensive research. Associations with the MHC class I and II

antigens have been conflicting, and the role of complement C2 and C4 deficiencies is not clear because of linkage disequilibrium with the HLA genes in the MHC. Many serological disorders are seen in lupus, but the cause of the autoantibodies remains elusive. Part of the problem in studying SLE is the extreme clinical diversity seen in the patients, which may be due to different genetic influences. It has been proposed the SLE is due to several factors: environmental, such as a virus; genetic predisposition, (one or more genes); and accelerating factors, such as estrogen (Alarcon-Segovia 1984).

Molecular genetic techniques now make it possible to analyse more closely the genetic susceptibility to SLE. Restriction fragment length polymorphisms (RFLPs) can be examined to look for unusual RFLPs in patients which do not occur in healthy individuals, or an increase in a particular RFLP which may reflect linkage disequilibrium with a susceptibility gene or locus. Genes for the MHC class II antigens have been cloned, so their contribution to SLE can now be studied at the DNA level. Many RFLPs in the HLA-D genes have been reported and DNA-DR typing is now possible also (Kohonen-Corish and Serjeantson 1986a). T-cell antigen receptor genes have been recently isolated, which permits examination of these genes, the products of which play an important role in the immune response.

This thesis firstly examines RFLPs in the class II DR, DQ and DP MHC genes, and applies the technique of DNA-DR typing to SLE patients, for whom serological DR typing is not always reliable (Chapter 3). The contribution of the class III complement components C2 and C4 to the pathogenesis of SLE is examined in three ethnic groups to look at the role of these complement deficiencies in SLE, and to compare the contribution of the class II and III genes to SLE, which has been a

problem in the past due to linkage disequilibrium (Chapter 4). T-cell receptor genes (α , β and γ) have been examined by RFLP analysis to look for unusual rearrangements or unusual clonality in these genes in patients with SLE (Chapter 5). Finally, in Chapter 6, Gm phenotypes, LCAs, RF and complement component C3 are studied in relation to SLE, and discriminant analysis of all the experimental data is performed to search for clinical subsets of SLE and to look for possible genetic combinations which could result in susceptibility to SLE.

In conjunction with this study on SLE, other related connective tissue diseases have also been examined to look for susceptibility genes in these diseases.

CHAPTER TWO

METHODS

2.1 INTRODUCTION.

The following methods describe the basic techniques used in the work of this thesis. Specific details of particular studies are included in the appropriate chapter.

2.1.1 PATIENTS AND CONTROLS.

Patients with SLE and related connective tissue diseases came from Sydney where they were reviewed by Dr. Paul Gatenby (Clinical Immunologist, Royal Prince Alfred Hospital). Patients with SLE were positive for four or more of the American Rheumatism Association Revised Criteria for SLE (Tan et al. 1982). Patients with the connective tissue diseases scleroderma (progressive systemic sclerosis or PSS), CREST, linear scleroderma, Primary Sjogren's syndrome, polymyositis (Pm), dermatomyositis (Dm), polymyositis/dermatomyositis (Pm/Dm), mixed connective tissue disease (MCTD), and undifferentiated connective tissue disease (UNCTD) were also studied. Patients with scleroderma were diagnosed according to the ARA preliminary criteria described by Masi et al. (1980), while diagnosis of the CREST subset of patients, characterized by anti-centromere antibodies, followed guidelines by Steen et al. (1984). The few patients studied with linear scleroderma were typical of the description by Falanga et al. (1986). Clinical features of polymyositis and dermatomyositis described by Bohan and Peter (1975) were used in the diagnosis of these patients. Patients with undifferentiated connective tissue disease included those with predominantly musculoskeletal features where a definite diagnosis could

not be made (Le Roy et al. 1980), while classic mixed connective tissue disease has been described by Sharp et al. (1972). Patients with Primary Sjogren's syndrome were diagnosed following guidelines by Fox et al. (1986).

In the analysis of experimental results, the following disease groups were combined because of low patient numbers: CREST, scleroderma and linear scleroderma; Pm, Dm and Pm/Dm; MCTD and UNCTD.

Controls comprised healthy unrelated blood donors from the Woden Valley Hospital Blood Bank in Canberra as well as healthy laboratory workers.

2.2 HLA-A, -B, -C, AND -DR SEROLOGICAL TYPING.

HLA-A, -B, -C, and -DR serological typing was performed using the standard complement dependent microcytotoxicity test. Heparinized blood (50ml) from the SLE and CTD patients was taken in Sydney with informed consent and transhipped overnight to Canberra. Heparinized blood (20ml) was taken from the controls, with informed consent, and was taken immediately to the Canberra laboratory where HLA typing was performed.

Whole blood was centrifuged at 2000 rpm for 10 min and the buffy coat layer of white cells harvested. This was diluted 1:2 with phosphate buffered saline (PBS; 2% by volume of 0.2M phosphate buffer pH 7.2 in 0.9% NaCl), underlaid with Ficoll-Paque (Pharmacia), and then centrifuged at 2000 rpm for 20 min. The white cells were harvested and washed with 10% fetal calf serum (FCS) (Flow Laboratories) in PBS, and then the platelets were removed with a 20% sucrose gradient at 1000 rpm for 10 min. The cells were washed again in 10% FCS in PBS and then the T-cells were separated by rosetting with AET treated sheep red blood cells (SRBC), (see below for preparation of AET treated SRBC). The

lymphocytes were suspended in 3 ml 10% FCS in PBS, and 3 ml of 1% AET treated SRBC were added. The white cell/SRBC mix was incubated at 37°C for 10 min, centrifuged at 2000 rpm for 10 seconds and then incubated at 4°C for at least 30 min, after which the cells were underlaid with Ficoll-Paque and centrifuged for 20 min at 2000 rpm. The non-rosetting cells could then be harvested leaving the rosetting T-cells in the bottom of the testtube with the SRBC.

2.2.1 T-CELLS.

T-cells were separated from the SRBC by incubation at 37°C for 10 min with 0.83% ammonium chloride in distilled water, which lyses red blood cells. The T-cells were centrifuged down and were then washed twice with 10% FCS in PBS, counted and diluted to a concentration of 2×10^6 cells/ml.

2.2.2 NON-ROSETTING CELLS.

The non-rosetting cells were harvested, washed, and resuspended in 10% FCS in PBS (2ml) and were then treated with 1% iron filings in PBS (2 ml) and a further cycle of AET SRBC (2 ml), incubating firstly at 37°C for ten minutes, centrifuging at 2000rpm for 10 seconds and then incubating at 4°C for at least 30 minutes to remove monocytes. The B-cells were isolated over a Ficoll-Paque gradient, centrifuging at 2000 rpm for 20 minutes. The B-cells were then washed twice in 10% FCS in PBS, counted and diluted to 2×10^6 cells/ml.

2.2.3 TISSUE TYPING.

T- and B-cells were loaded onto locally prepared trays, with 60 wells each, which contained 1 μ l of antisera in each well, covered in paraffin oil (Sigma). These trays were stored at -20°C until they were used. T- or B-cells (1 μ l per well) were loaded onto the trays which were then incubated at about 22°C for 30 min for the HLA-A, -B, and -C typing and 60 min for the HLA-DR typing. Rabbit complement from Pel Freeze (5 μ l) was then added and the HLA-A, -B, and -C trays were incubated for a further 60 min and the HLA-DR trays for two hours. The cells were stained with a 5% eosin yellow dye (1 μ l/well) and were fixed with formalin (2 μ l/well). The trays were then placed at 4°C overnight to allow the cells to settle and were read the next day with an inverted light microscope. Most of the antisera used was obtained locally from Canberra, or regionally (from Australia). Some of the patients were typed with antisera from the Third Asia-Oceania Histocompatibility Workshop.

2.2.4 AET TREATMENT OF SHEEP RED BLOOD CELLS.

Sheep red blood cells were treated with 2-aminoethylisothioronium bromide hydrobromide (AET, from Sigma) by a modified method of Pellegrino et al. (1975). Sheep blood was collected into Alsevers solution and the RBC were then washed five times with normal saline. Packed SRBC (2ml) were mixed with 10ml of 2% AET in distilled water, pH 8.0, and incubated for 15 minutes at 37°C, inverting the testtube gently every five minutes to mix the cells. The AET treated SRBC were then washed five times in normal saline. The AET SRBC were stored at 4°C for up to two weeks after which time they were discarded. A 1% solution of

the AET SRBC in FCS was used for rosetting.

2.3 DNA EXTRACTION, DIGESTION, AND SOUTHERN BLOTS.

2.3.1 DNA EXTRACTION.

Genomic DNA was extracted from buffy coat white cells harvested after the first Ficoll-Paque gradient during HLA typing. The buffy coat was frozen at -20°C until DNA was extracted. The buffy coat was washed three times with T_{20}E_5 , resuspended in 4ml of the same buffer, and incubated overnight at 37°C with 200 μl 10% SDS and 400 μl Proteinase K from Sigma (10 mg/ml). The suspension was extracted twice with an equal volume of Tris pH 8.0 equilibrated phenol:chloroform:iso amyl alcohol (25:24:1), and then twice with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA was then ethanol precipitated overnight at -20°C with 5M NaCl. The precipitated DNA was centrifuged down, air dried and resuspended in T_{10}E_1 to a concentration of about 0.3 mg/ml

2.3.2 DNA DIGESTION.

The following procedure was used in the digestion of DNA samples:

30 μl (10 μg) DNA
5 μl 10x core buffer
x μl restriction enzyme
x μl distilled water to 50 μl .

Core buffers were made according to manufacturers recommendations at ten times the digestion concentration and were stored at -20°C in small

aliquots. The restriction enzymes used were BamHI (Boehringer-Mannheim); BglII (Pharmacia); EcoRI (Boehringer-Mannheim); EcoRV (Boehringer-Mannheim); TaqI (Biolabs); and PvuII (Pharmacia). All digests were performed at 37°C overnight with the exception of TaqI digests which were done at 65°C for 2 hours.

Digestions were stopped with 2.5µl 0.5M EDTA and the DNA ethanol precipitated with 2M NaOAc (5µl 2M NaOAc, 110µl ethanol) and placed in dry ice for about 30 min. (The digested DNA was sometimes precipitated at -20°C overnight or longer). The DNA was centrifuged down and air dried for about 30 min and was then resuspended in 16µl of T₁₀E₁. Ficoll/dye buffer containing bromophenol blue and xylene cyanol (Maniatis et al. 1982) was added, the samples heated to 65°C for 5 min and then placed on ice and loaded onto a gel, which was submerged in buffer. The DNA was fractionated on a 0.8% agarose gel in TAE buffer, (0.04M Tris-Acetate, 0.001M EDTA pH 8.0), using submarine apparatus (Pharmacia, GNA-200). Gels were 20cm by 20cm and 7mm thick, with 22 wells which held about 25µl of solution. The DNA was run out of the wells at 90V for about 30 min and the voltage was then set at 20V overnight. The bromophenol blue dye had usually migrated about 11cm when electrophoresis was stopped. The gels were stained with ethidium bromide for about 20 minutes and photographed under UV light with a red filter using a Polaroid camera.

2.3.3 SOUTHERN BLOTS.

The gels were blotted by the method of Southern, (1975) or Reed and Mann (1986). With Southern's method, the gels were washed at room temperature by gentle shaking for 25 min with 0.25M HCl to depurinate the DNA, for 40 min with 0.4N NaOH, 0.6M NaCl to denature the DNA and then for 40 min with 1.5M NaCl, 0.5M Tris pH7.5 to neutralise. The gels

were blotted onto GeneScreen Plus membranes (New England Nuclear) with 10xSSC overnight (1xSSC= 150mM NaCl, 15mM trisodium citrate). The membranes were rinsed in 0.4M NaOH for 30 secs and 2xSSC, 0.2M Tris pH 7.5 for 30 secs and allowed to air dry completely. With Reed and Mann's method, the gels were washed in 0.25M HCl for 25 min and were then blotted onto Genescreen Plus membranes with 0.4M NaOH overnight. The filters were then rinsed in 2xSSC, 0.2M Tris pH 7.5 for 30 secs and allowed to air dry completely.

2.4 PREPARATION AND NICK TRANSLATION OF THE PROBES.

cDNA probes were prepared following the SDS method described by Maniatis et al. (1982). Probes were purified by a CsCl gradient (Maniatis et al. 1982).

Probes were nick translated using the Amersham nick translation kit, following the manufacturers recommendations. The following reaction mix was used:

- 0.25 to 1µg of probe
- 10µl nucleotide buffer
- 3µl to 12µl α -³²P dATP or α -³²P dCTP
- 5µl enzyme
- xµl distilled water to 50µl

A maximum of 1µg of DNA was nick translated in a particular reaction mix, with 3µl of radioactive label used per 0.25µg of DNA. The whole plasmid containing the probe was used in the nick translation. The reaction mixture was mixed well before being incubated at 15°C for 60 minutes, after which time 2.5µl of 0.5M EDTA was added to stop the reaction. The labelled probe was separated from the unincorporated

radioactive label by passing the reaction mix through a 5ml column of Ultrogel (AcA54, LKB) with $T_{10}E_1$. The labelled probe was collected in an eppendorf. If the incorporation of the radioactive label was too low, the probe was discarded.

2.5 HYBRIDIZATIONS.

Hybridizations were performed following the method of Nasmyth (1982) with a few modifications. Filters were placed in plastic freezer bags (2 per bag, DNA side out) and were prehybridized in 15ml of prehybridization solution for at least 30 min at room temperature. The prehybridization solution was prepared in the following way. Distilled water (6.8ml or 0.46 total volume) was boiled for ten minutes with sonicated salmon sperm DNA (50 μ g/ml), and was then chilled on ice after which 8.1ml of Nasmyth solution was added. The tube was inverted to mix the solution and was then added to the bag with the filters making sure that the filters were completely wet and there were no air bubbles. Nasmyth solution contained 1.1M NaCl, 0.333M Na_2HPO_4 , 0.0111M EDTA pH 6.2, 1.85% sodium lauroyl sarcosine and 18.5% dextran sulphate. Prior to hybridization, any excess prehybridization solution was squeezed out of the bag. Hybridizations were carried out in a solution prepared the same way but containing 1×10^8 cpm/ μ g of ^{32}P labelled probe which was boiled with the water and salmon sperm DNA. For every 100cm² of filter, 10ml of solution was used with 0.1 μ g of probe. The hybridization fluid was sometimes reused for a second set of filters, with the whole hybridization solution boiled for 10 minutes, cooled briefly on ice, and then put into the hybridization bag which was sealed after all the air bubbles were removed. Hybridizations were carried out at 65°C for 22 to 44 hours in a shaking water bath.

Filters were washed at room temperature for 5 min in 2xSSC, 0.1% SDS; at 65°C for 30 min in 1xSSC, 0.1% SDS; and at 65°C for 30 min in 0.5xSSC, 0.1% SDS. The filters were then usually at 5 to 10 cps but if not they were given a second wash in the 0.5xSSC, 0.1% SDS buffer at 65°C for 15 to 30 min. Filters were allowed to air dry (not completely) for 5 to 10 min and were then wrapped in Glad Wrap. Autoradiography was carried out using Fuji Xray film and Lightning Plus intensifying screens (Du Pont) in cassettes at -70°C for 3 to 7 days. The films were developed with an automatic film developer.

2.5.1 REHYBRIDIZATIONS.

The GeneScreen Plus membranes were reused several times with excellent results. Probes were stripped from membranes according to manufacturers recommendations. The filters were washed in 0.4M NaOH at 42°C for 30 minutes with a shaking water bath, and then in 0.1xSSC, 0.1% SDS, 0.2M Tris pH7.5 at 42°C for 30 minutes. Membranes were then air dried for five to ten minutes. The membranes were reused with prehybridizing and hybridizing carried out as before.

2.6 C4 ALLOTYPING.

C4 typing was performed by the method of Awdeh and Alper (1980) and as described by Blake and Radford (1984).

2.6.1 MATERIALS AND METHOD.

- | | |
|---|--------|
| 1. 0.2M EDTA pH 7.2: Na_2EDTA | 37.22g |
| Na_4EDTA | 38.40g |
| Distilled water to 1L | |

This solution was stored frozen at -20°C , with a working aliquot kept at 4°C for no longer than one week.

- | | |
|---|-------|
| 2. Dialysis stock solution: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ | 41.8g |
| $\text{NaHPO}_4 \cdot \text{H}_2\text{O}$ | 33.1g |
| NaEDTA | 12.5g |
| Distilled water to 1L | |

The pH of this solution was about 6.8. This stock solution was diluted 1:5 with distilled water, with one litre of the diluted dialysis solution was for each dialysis. The stock solution was kept no longer than one week.

- | | |
|-----------------------|---------|
| 3. Tank buffer: Tris | 22.60 g |
| Glycine | 28.10 g |
| Na barbiturate | 6.55 g |
| Barbituric acid | 1.035g |
| Distilled water to 1L | |
| pH 8.8 | |

- | | |
|--|---------|
| 4. Gel solution: Seakem ME (EEO 0.16-0.19) agarose | 0.75g |
| Tank buffer | 16.0 ml |
| 0.2 M EDTA | 1.6 ml |
| Distilled water | 22.4 ml |

The gel solution was boiled until the agarose was completely dissolved and then poured into the gel mould which was made as follows. A piece of Gel Bond (24.3cm x 12.5cm) (FMC Marine Colloids Division) was put on a glass plate with a little water on the hydrophobic side to bind it to

the plate. A second glass plate (preferably siliconized) was placed on top of the Gel Bond using a 1mm silicone gasket to separate the two plates. The plates were held together using bulldog clips. When the gel was set it was wrapped in Glad Wrap and placed at 4⁰C overnight. Samples (10µl of plasma) were desialized with neuraminidase (2µl) from Clostridium perfringens (Type VI, Sigma, St. Louis, MO, USA) by placing them on cellophane which had been boiled for at least 6 hours to develop hydrophobic properties. The cellophane was placed on top of thin towelling which had been soaked in the dialysis buffer. One end of the towel was in the buffer, while the other end was hanging over the edge of the container so that the dialysis buffer could move through the towel. Dialysis was carried out at 4⁰C overnight.

Excess water was removed from the gel where the samples were to be loaded using a piece of Whatman No.1 and the sample applicator strip (made from a piece of Gel Bond with slots 8-10mm long, 0.75mm wide and 2mm apart) was placed on the gel. Desialized plasma samples (10µl) were loaded into the wells and allowed to soak in for about 30 min before the applicator strip was removed and electrophoresis was started.

Haemoglobin was used as a marker to check how far the samples were moving down the gel. Samples were electrophoresed using LKB 2117 multiphor equipment, with the current running from the cathode to the anode. The gels were electrophoresed for 4 to 5 hours at 80 mA at 6⁰C, and were then overlaid with anti-C4 antiserum (Silenus, Australia) for 1 hour at room temperature under a glass dish to prevent dessication. The gels were then washed gently with tap water, squashed for 10 min and soaked in 0.9M saline overnight. The gels were washed again, pressed and air dried and stained with Coomassie Brilliant Blue. The gels were destained with the same solvent as used in the stain (9 parts methanol, 2 parts glacial acetic acid, 9 parts distilled water).

2.6.2 HAEMOLYTIC OVERLAY.

A haemolytic assay was performed when C4A and C4B alleles overlapped or when it was difficult to assign unusual alleles.

1. Complement Fixation Diluent (CFD). This was made with Oxoid complement fixation test diluent tablets (Code BR16). This makes a veronal buffered saline solution, pH 7.2.
2. Antibody coated sheep red blood cells. SRBC (10ml) collected into Alsevers solution was washed three times with PBS and the packed SRBC then made up to a 10% solution. For 1ml of 10% SRBC, 1ml EDTA-PBS (0.01M EDTA pH 7.2 in PBS) and 10µl rabbit anti-sheep antibody (Wellcome) was added. The SRBC were then incubated at 4°C for 10 minutes, washed once with EDTA-saline and twice with CFD. The SRBC were then reconstituted to a 10% solution in CFD.

After electrophoresis, a border was made around the edge of the gel with strips of Whatman (5MM), and overlay containing 7.5ml 2xCFD, 7.5ml 2% agarose, 750µl antibody coated SRBC, and 750µl C4 deficient serum from guinea pigs, was poured evenly over the surface of the gel. The gel was then placed at 37°C for 15 to 30 minutes to allow haemolysis.

C4 types were assigned according to the recommendations of Mauff et al. (1983). C4 gels were read independently by myself and another person.

2.7 C2 ALLOTYPING.

C2 phenotyping was performed as described by Blake and Radford (1984).

2.7.1 MATERIALS AND METHOD.

1. Acrylamide stock solution:

Acrylamide	3.35g
N,N-methylene-bis-acrylamide	0.90g
Taurine	16.68g
Distilled water to 500 mls.	

The solution was filtered and stored at 4°C.

2. Gel solution:

Acrylamide stock solution	26.5ml
Ampholyte (LKB ampholine) pH 5-7.	1.5ml
Riboflavin (1mg/100ml)	7.0ml

3. Iodine solution: Potassium Iodide	8.30g
Iodine	0.25g
0.5M phosphate buffer pH 6.0	40.0ml
Distilled water to 100 ml	

4. Complement fixation diluent (CFD) and antibody coated SRBC were made as described for C4 allotyping.

5. Haemolytic overlay: 2 x CFD	7.50ml
2% agarose	7.50ml
10% antibody coated SRBC	0.75ml
C2 deficient serum	0.20ml

A gel mould was made from a 1mm thick glass plate 16.5cm x 21.5cm and another glass plate 3mm thick, 19cm x 21.5cm. The plates were separated by a 1mm thick silicone gasket which was positioned about 0.5cm from the edge of the glass plates (the two long sides and one of the short sides). The plates were clamped together with bulldog clips

and the mould was stood up vertically with the open end uppermost. The gel solution was degassed with a vacuum pump and then poured into the mould. The gel was left to polymerize under a fluorescent light for 3 to 4 hours.

Strips of 20mm x 5mm Whatmann 3MM chromatography paper were placed at the anodal end of the gel and 20 μ l of plasma was applied to each strip. The gel was isoelectrofocussed using the apparatus described by Awdeh (1968), inverting the gel and placing it over carbon rod electrodes so it was in continuous contact with the electrodes. The gel was isoelectrofocussed at 4 $^{\circ}$ C for about 18hrs with power at 1.5 watts per gel, and a maximum voltage of 1000 volts per gel with the initial current at 6mA per gel which dropped to about 2mA per gel when the gel was focussed. Gels were soaked in the iodine solution for 30 min at room temperature and then in 1xCFD for 30 min also at room temperature. A barrier was formed around the edge of the gel using strips of 5MM Whatmann chromatography paper and the haemolytic overlay solution was poured over the surface of the gel. The gel was then placed in a sealed container to prevent dessication and incubated at 4 $^{\circ}$ C for about 60 min, and then at 37 $^{\circ}$ C for 2 to 4 hours until bands of haemolysis appeared. The gel was read by myself and another person.

2.8 C2 DEFICIENCY ASSAY.

The C2 deficiency assay was performed by the radial diffusion assay according to the method described by Lachmann and Hobart (1978). Normal human serum was warmed to 37 $^{\circ}$ C and 50 μ l of the yeast preparation (Lachmann and Hobart 1978) was added. The serum was then shaken at 37 $^{\circ}$ C for 45 min and centrifuged to remove the yeast. Antibody coated SRBC (1ml) were diluted to 10ml with 1xCFD and warmed to 37 $^{\circ}$ C when one ml of

the yeast treated serum was added. The mixture was stirred at 37⁰C for 70 sec and then 0.2ml of suramin (Antrypol, B.P. Bayer 205) at 50 mg/ml was added. This was shaken gently at 37⁰C for five min and the SRBC were then spun down and washed three times with 1xCFD. The SRBC were then resuspended in 1ml CFD. C2 was destroyed by incubating the SRBC in CFD/sucrose (9%) at 37⁰C for 90min. The red blood cells were washed in CFD/sucrose twice after this treatment. A gel was made by mixing the following:

7.5ml 2% agarose (56⁰C)

7.5ml 2x CFD/18% sucrose

100µl C2 reagent (Lachmann and Hobart 1978)

1.0ml Treated SRBC

which was then poured onto a glass slide (12cm x 12cm) which had been warmed to about 37⁰C. When the gel was set, holes 2 mm in diameter were cut in the gel which were filled with 6ul of test sera at the following dilutions: 1:1, 1:4, and 1:8. The gels were incubated overnight at 4⁰C and then placed at 37⁰C the next day for one to two hours. Clear rings of lysis were then visible and were measured against a control who was known to have either 100% or 50% levels of C2.

2.9 C3 TYPING.

C3 typing was carried out following the method of Teisberg et al. (1970).

2.9.1 MATERIALS AND METHOD.

1. Gel buffer: 0.0230M NaBarbitol

0.0037M Barbitone

0.0009M CaLactate

Distilled water to 30 ml (gives pH 8.65)

2. Bridge buffer: 0.0610M NaBarbitol

0.0106M Barbitone

0.0018M CaLactate

Distilled water to 1L (gives pH 8.65)

A gel was made by mixing 0.3g agarose and 30ml gel buffer and boiling to melt the agarose. This was then poured onto the surface of a warm glass plate (17.2cm x 15.2cm) and allowed to set underneath a glass dish to prevent dessication. Wells were cut about 2cm from the cathodal end of the gel using a plastic template, and were blotted to take up any moisture from the slots. Plasma (5µl) was loaded into the wells, and plasma + bromophenol was used as a marker in one well and a control of known C3 type FS was also used in one well. Gels were electrophoresed for about 10 min at 25mA, and the wells were then sealed with some left over gel solution and the gel electrophoresed at 480V for 2 to 3 hours. Cooling plate electrophoresis equipment was used.

The gels were stained according to Johansson et al. (1972). The gels were immersed in saturated picric acid:glacial acetic acid (5:1) for 15 minutes to fix the protein. The gels were then rinsed in running tap water for 2-3 minutes. They were then covered with a sheet of wet Whatmann, (No. 1), and several sheets of paper towelling and blotted with a 2-3kg weight for 10 minutes. The gels were air dried with a fan until the agarose became clear and were then stained with Coomassie Blue (0.2%) in methanol: glacial acetic acid: distilled water (9:2:9) which

was filtered before use. The gels were destained in 2.5% acetic acid, 2.5% Teepol in water. The gels were read independently by myself and another person.

2.10 TESTING FOR LYMPHOCYTOTOXINS.

Lymphocytotoxins against both B- and T- cells were examined using the complement dependent microcytotoxicity test as described for the HLA-A, -B, -C, and -DR serological typing. B- and T-cells were isolated from healthy controls of known HLA type in the same way as for tissue typing, and were tested against SLE sera. The trays were incubated at both 4⁰C and 15⁰C initially to see which temperature gave the best result. The lymphocytotoxins were identified equally well at both temperatures so the experiments were then carried out at 15⁰C. The trays were read in the same way as for normal tissue typing.

2.11 STATISTICAL ANALYSIS.

Significance was tested for by the chi square statistic (Armitage 1971) using 2x2 contingency tables. When any number in a 2x2 cell fell below 5, Fisher's exact probability was calculated (Armitage 1971). Statistical methods specific for particular parts of this thesis are outlined in the appropriate chapter.

CHAPTER THREE

DR GENOTYPING IN SLE AND OTHER MHC CLASS II RFLPS

3.1 INTRODUCTION.

3.1.1 HLA-DR TYPING IN SLE.

Serological HLA-DR typing is notoriously difficult in patients with SLE due to multiple autoantibodies including anti-lymphocyte antibodies (Terasaki et al. 1970) and low levels of circulating B-lymphocytes (Gladman et al. 1979), which are often of poor viability (Stastny 1978). Difficulties in HLA-DR typing are further exacerbated when patients receive immunosuppressive therapy (Hokland et al. 1981; Snyder and Unanue 1982). In published reports of HLA-DR profiles in SLE patients, technical problems in HLA-DR typing have seldom been discussed, although one leading histocompatibility laboratory failed to achieve HLA-DR typing in as many as 21% of patients tested (Gladman et al. 1979).

When a high proportion of any series cannot be DR typed, there is potential for bias in that some HLA-DR antigens may not be as confidently typed as others. This phenomenon may well contribute to the conflicting findings regarding the particular HLA-DR antigens at risk in SLE. HLA-DR2 has been implicated in SLE in some studies of Caucasoids (Reinertsen et al. 1978; Reveille et al. 1985) but not in others (Scherak et al. 1979; Celada et al. 1979). Similarly, HLA-DR3 has often been associated with an increased risk for SLE (Reinertsen et al. 1978; Reveille et al. 1985) but not consistently (Ahearn et al. 1982).

Unequivocal HLA-DR genotypes can now be established by the application of recombinant DNA techniques in healthy individuals (Owerbach et al. 1983; Spielman et al. 1984) as well as in patients with chronic myelocytic leukaemia (Hui et al. 1985) or with the form of combined immunodeficiency syndrome (Marcadet et al. 1985) where HLA-DR

antigens are not expressed. For instance, using the restriction endonuclease TaqI and a DR β chain gene probe, each serological DR type from DR1 to DRw10, with the exception of DR3 and DRw6, gives a unique and characteristic fragment pattern after hybridization (Kohonen-Corish and Serjeantson 1986a; Kohonen-Corish et al. 1986). DR3 and DRw6 show similar restriction fragment length polymorphisms (RFLPs) and these two DR types are differentiated by their linkage disequilibrium relationships with DQ α (Spielman et al. 1984; Trowsdale et al. 1985) and DQ β (Kohonen-Corish and Serjeantson 1986a) TaqI fragments. By using DR β , DQ α , and DQ β gene probes with TaqI digested DNA, some DR types can be subdivided by RFLP patterns that correlate with HLA-D, including DR2 (Dw2, Dw12, and Dw"AZH") and DRw6 (Dw9, Dw18, and Dw19). Further, some DR types such as DR3, DR5, and DR7 show greater heterogeneity at the genomic level than serologically which is useful in studies of HLA related diseases and also in population studies.

HLA-DR and -DQ RFLPs have been extensively described in various populations in Asia-Oceania (Kohonen-Corish and Serjeantson 1986b; Kohonen-Corish et al. 1988) which gives the opportunity to examine HLA-DR profiles in SLE in other populations as well as in Caucasoids, and also to see if there are any common RFLP patterns in SLE patients of different ethnicities. Studies of HLA-DR profiles in Japanese SLE patients have been conflicting as they are in Caucasoid populations. The HLA-DR2 antigen frequency was increased in SLE patients in one study (Hashimoto et al. 1985), but in another, there was an increase of DR4 (Sakurami et al. 1982). In a third study, HLA-DR4 was decreased in frequency (Kameda et al. 1982).

3.1.2 HLA-DR AND HLA-DQ IN CONNECTIVE TISSUE DISEASES.

Few HLA-DR studies have been performed on the lupus like connective tissue diseases and similar problems could be expected in HLA-DR typing these patients as are seen in SLE. Scleroderma has been associated with HLA-DR5 in some studies (Gladman et al. 1981; Briggs et al. 1986) and showed a non-significant increase in a third (Mollenhauer et al. 1984), but no associations were seen in two other studies (Lynch et al. 1982; Niks et al. 1982). It has been suggested that HLA-DR5 is also increased in the CREST form of scleroderma (Briggs et al. 1986). One study with 12 patients with polymyositis reported an increase in HLA-DR3 (Hirsch et al. 1980) while primary Sjogren's syndrome has shown associations with HLA-DR3 and DQw2 (Wilson et al. 1984). Harley and colleagues (1986) have reported an interactive effect between DQw1 and DQw2 in Sjogren's syndrome patients. Because of the nature of these connective tissue diseases and the immunosuppressive therapy that many patients receive, HLA-DR typing in these diseases could have been affected as it has been in SLE.

3.1.3 HLA-DQ AND HLA-DP GENES IN SLE.

Few studies of SLE have examined the role of the MHC DQ genes in the disease. One large study by Ahearn et al. (1982) with 60 white SLE patients showed an increase in DQw1 compared with controls from the Eighth International Histocompatibility Workshop ($p < .005$), but the increase was not significant when local controls were used. The authors suggested that the HLA genes were not the primary susceptibility genes but could have had some effect on disease expression. Isolation of the

DQ α (Schenning et al. 1984) and DQ β (Long et al. 1982) genes allows more detailed analysis of these genes in relation to SLE. Although many DQ α and DQ β RFLPs show linkage to DR types, some can be used in disease analysis, and, as is the case for insulin dependent diabetes mellitus, may show closer disease associations than the DR genes (Todd et al. 1987).

There have been no published reports investigating the HLA-DP genes in SLE. Until recently, primed lymphocyte typing was the only method available to study the DP antigens (Shaw et al. 1980), but monoclonal antibodies are now becoming available. A major study of serological DP typing has only just been undertaken as part of the Tenth International Histocompatibility Workshop. Since the DP α and DP β genes have been isolated, a few studies on RFLPs in the DP region have been published with some promising results in the area of DNA-DP typing (Hyldig-Nielsen et al. 1987; Bodmer et al. 1987).

3.1.4 AIMS.

The present study examines potential bias in serological HLA-DR typing of SLE patients by probing TaqI digests of genomic DNA with probes of HLA-DR β , DQ α , and DQ β chain genes to establish HLA-DR genotypes in Caucasoid patients. These results are compared with DR phenotypes determined by conventional tissue typing techniques. The HLA-DR antigen profile as determined by DNA-typing is compared with that in appropriate control populations. In addition, HLA-DR profiles in Japanese SLE patients are examined by DNA-DR typing. Patients with various lupus like CTDs have also been DNA-DR typed and their antigen profiles are presented.

HLA-DQ types have been assigned by DNA typing so that DQ distributions can be examined in SLE and the connective tissue diseases. Further, The HLA-DP β genes have been analysed by RFLP studies in both the SLE and CTD patients.

3.2 MATERIALS AND METHODS.

3.2.1 PATIENTS AND CONTROLS.

Caucasoid patients with SLE and the connective tissue diseases scleroderma, CREST, primary Sjogren's syndrome, polymyositis/dermatomyositis (Pm/Dm), mixed connective tissue disease (MCTD), and undifferentiated connective tissue disease (UNCTD) were reviewed in Sydney by Dr. P. Gatenby and blood was sent overnight to Canberra where serological HLA class I and II typing was performed. Buffy coat white cells were separated and stored frozen at -20°C until DNA was extracted.

Caucasoid controls comprised 39 random blood bank donors from Canberra and 30 healthy laboratory personnel.

Japanese SLE patients were reviewed by Dr. S. Naito and were examined serologically for HLA class I and II antigens as part of the Third Asia Oceania Histocompatibility Workshop (Naito et al. 1986). Buffy coats from the patients and controls were sent frozen in dry ice to Canberra where DNA was extracted for DNA-DR analysis.

3.2.2 SEROLOGICAL HLA CLASS I AND II TYPING.

All the Caucasoid patients were HLA-A, -B, and -C typed and a

random subset of these were also HLA-DR typed serologically as described in Chapter 2. The blood bank controls and some of the laboratory personnel were HLA-A, -B, -C, and -DR typed serologically while the remaining controls were typed only for HLA class I antigens.

The Japanese patients and controls were typed for HLA-A, -B, -C, and -DR antigens in Japan by Dr. S. Naito.

3.2.3 DNA-DR TYPING.

Genomic DNA was prepared from the patients and controls and was then digested with *TaqI* and transferred to GeneScreen Plus membranes as described in Chapter 2. The filters were hybridized with probes for *DR β* , *DQ α* , and *DQ β* following the protocol in Chapter 2. *DR β /TaqI* RFLP associations with DR antigens have been described (Kohonen-Corish and Serjeantson 1986a; Serjeantson et al. 1986) and the most common Caucasoid RFLP *DR β /TaqI* patterns and their corresponding DR types are summarised in Table 3.1. *DQ α /TaqI* RFLPs (Trowsdale et al. 1985) and *DQ β /TaqI* RFLPs (Kohonen-Corish and Serjeantson 1986a) correspond with DR types due to linkage disequilibrium, and the DQ and DR associations are presented in Table 3.2. *DQ β /BamHI* RFLPs also correlate with DR types (Owerbach et al. 1983) and the 3.7kb and 12kb fragments can be used in subsetting some DR types (Kim et al. 1985) and these are also shown in Table 3.2.

3.2.4 OTHER HLA CLASS II HYBRIDIZATIONS.

SLE and CTD patients were examined for *DR α* and *DP β* RFLPs following the methods described in Chapter 2.

TABLE 3.2 DQ RFLPs AND THEIR DR ASSOCIATIONS IN CAUCASOIDS.

		TaqI/DQα (kb) ^a					TaqI/DQβ (kb)					BamHI/DQβ (kb) ^b		
DQ type	DR type	6.8	6.2	5.7	5.0	3.8	2.9	6.7	5.5	4.6	3.5	3.0	12.0	3.7
DQw1	DR1					+	+	+		+	+			
	DR2.Dw2		+			+				+	+	+		
	DR2.Dw12		+			+				+	+			
	DR2.KAS11		+			+		+		+	+			
	DRw6.Dw18		+			+				+	+	+		
	DRw6.Dw19		+			+		+		+	+			
	DRw6.Dw9					+	+	+		+	+			
DRw10					+	+	+		+	+				
DQw2	DR3s				+	+				+	+			
	DR31				+	+				+	+			
	DR7			+		+		+		+	+			
DQw3	DR4.TA10+			+		+				+	+		+	
	DR4.TA10-			+		+				+	+		+	
	DR5				+	+				+	+			+
	DR7.B57			+		+				+	+		+	
	DRw8					+	+			+	+		+	
	DRw9			+		+	+			+	+		+	

a DX RFLPs of 2.2 and 2.1kb seen with DQα/TaqI not included

b only BamHI 12kb and 3.7kb RFLPs included

TABLE 3.3 SOURCE AND DESCRIPTION OF PROBES.

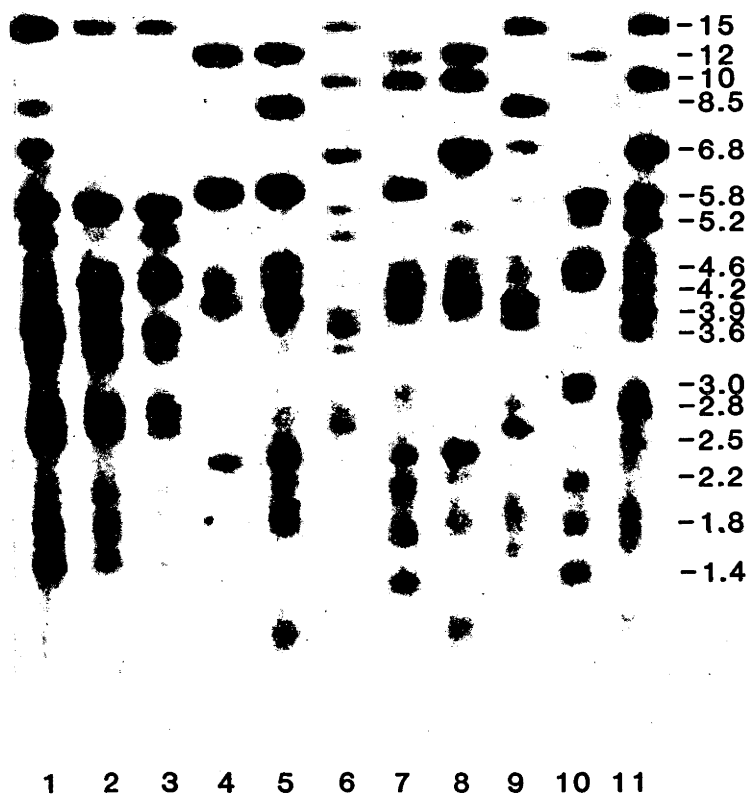
<u>PROBE</u>	<u>DESCRIPTION</u>	<u>REFERENCE</u>	<u>SOURCE</u>
DR α	1100bp in pBR322	Stetler <u>et al.</u> 1982	Dr. H. Erlich, Cetus Corporation
DR β	1300bp in pBR322	Long <u>et al.</u> 1982	Dr. B. Mach, University of Geneva
DQ α	500bp in pBR322	Schenning <u>et al.</u> 1984	Dr. D. Larhammar, University of Uppsala
DQ β	1250bp in pBR322	Long <u>et al.</u> 1982	Dr. B. Mach, University of Geneva
DP β	50,100,and 700bp	Gustafsson <u>et al.</u> 1984	Dr. D. Larhammar, University of Uppsala

TABLE 3.4 PROBE/ENZYME COMBINATIONS USED.

<u>PROBE</u>	<u>ENZYME</u>	<u>REFERENCE</u>
DR α	BglII EcoRV	Stetler <u>et al.</u> 1982 Erlich and Stetler 1984
DR β	TaqI	Kohonen-Corish and Serjeantson 1986a
DQ α	TaqI	Trowsdale <u>et al.</u> 1985
DQ β	TaqI BamHI	Kohonen-Corish and Serjeantson 1986a Owerbach <u>et al.</u> 1983
DP β	BglII EcoRV	Kohonen-Corish 1987 Kohonen-Corish 1987

FIGURE 3.1: DR β hybridization of TaqI digested DNA from SLE patients.
DR types are indicated at the top of each lane.

DR 4,7 1,9 1,4 5,- 5,8 3s,4 2,5 3s,6l 7,8 1,2 6s,4



3.2.5 PROBES.

The probes used in this study are shown in Table 3.3, and the probe/restriction enzyme combinations are given in Table 3.4.

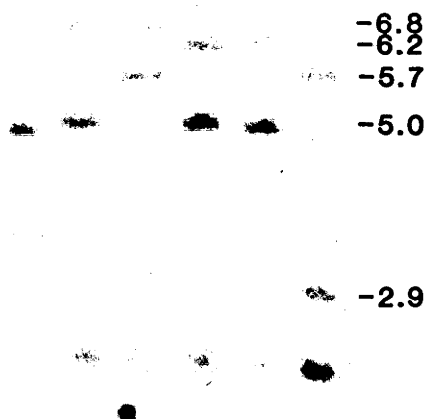
3.3 RESULTS.

3.3.1 DNA-DR TYPING.

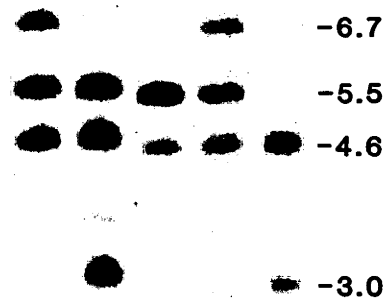
An autoradiograph of genomic DNA from SLE patients digested with TaqI and probed with DR β is shown in Figure 3.1. The results clearly show the DR types of the patients, with the characteristic DNA fragments associated with each DR type. Only HLA-DR3 and -w6 cannot be distinguished by DR β /TaqI hybridizations, but their linkage relationships with DQ α and DQ β can be used to differentiate between them and to assign Dw types. Hybridizations of TaqI digested DNA probed with DQ α or DQ β are shown in Figures 3.2 and 3.3 respectively. The DRw6 subtype DRw13 has either a 6.8 (Dw18) or 6.2kb (Dw19) DQ α /TaqI fragment while DRw14 (Dw9) has an alternative 2.9kb fragment; DR3 has a characteristic 5.0kb TaqI/DQ α fragment which it shares with DR5. These linkage relationships have already been described by Trowsdale et al. (1985), who assigned slightly different sizes to the DNA fragments. Similarly, DRw6 positive cells have either a 5.5kb (Dw19) or 3.0kb (Dw18) DQ β /TaqI fragment which is absent from DR3 positive cells (Kohonen-Corish et al. 1986a). DR2 can also be subdivided by its linkage relationships with DQ α and DQ β TaqI RFLPs, such that Dw2 has a 6.2kb DQ α /TaqI fragment and a 3.0kb DQ β /TaqI RFLP while Dw12 has a 6.8kb DQ α /TaqI fragment and the monomorphic DQ β /TaqI fragments. The Dw"AZH" specificity in Caucasoids

FIGURE 3.2: a. $DQ\alpha$ hybridization of $TaqI$ digested DNA.
b. $DQ\beta$ hybridization of $TaqI$ digested DNA.
c. $DQ\beta$ hybridization of $BamHI$ digested DNA.

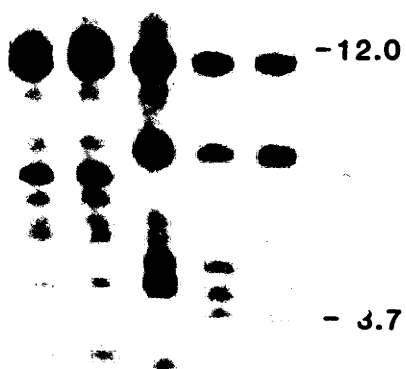
a. DQ α /TaqI



b. DQ β /TaqI



c. DQ β /BamHI



has a 6.2kb DQ α /TaqI and a 5.5kb DQ β /TaqI fragment. One variant of DR2 does not have the usual DR β /TaqI RFLPs associated with DR2 but instead has four fragments, 15kb, 4.4kb, 2.2kb, and 1.7kb in size. This DR2 pattern has also been seen in the Tenth International Histocompatibility Workshop cell line called KAS011 (Dr. S. Serjeantson, personal communication). This DR2 variant was seen both in the Caucasoids and Japanese examined here, but was not common.

3.3.2 RELIABILITY OF SEROLOGICAL DR TYPING IN SLE.

A sample of 39 of the total 69 controls typed at the DNA level as well as by traditional serological techniques showed excellent correlation ($r=0.99$, $p<.01$) between DNA-DR typing and serological DR antigen assignment. One error occurred, in the non-assignment of a DR5 antigen. The excellent correspondence between DR β chain RFLPs and HLA-DR types in controls confirms the reliability of DNA typing and the suitability of applying this technique in SLE patients. There were no TaqI DR β fragments seen in the patients that did not occur in the controls.

The correlation of serological DR assignment with DNA-DR assignment in SLE patients is shown in Table 3.5. It can be seen that 35% of DR antigens in SLE patients could not be assigned at all. The success of serological antigen assignment was non-random with DR2 and DR3 more readily typed than DR5 and DRw6(w13). These differences were statistically significant. The chi-square value for ease of typing DR2 compared with DRw13 was 6.8 ($p <.01$) and compared with DR5 was 6.0 ($p <.05$).

Similarly DR3 was significantly more readily assigned than DR5 or

TABLE 3.5 CORRELATION OF SEROLOGICAL DR TYPING WITH
DNA-DR TYPING IN SLE.

DNA-DR Assignment		Serological DR antigen assignment Correlation with DR type (%)		
Antigen	Number	Agree	Disagree	No result
DR1	10	60.0	20.0	20.0
DR2	9	77.8		22.2
DR3	27	77.8	3.7	18.5
DR4	16	56.3		43.6
DR5	7	14.3	28.6	57.1
DRw13	11	18.2	18.2	63.6
DRw14	1	0.0	100.0	0.0
DR7	10	60.0		40.0
DRw8	0	-	-	-
DRw9	1	0.0		100.0
DRw10	0	-	-	-
Total	92	56.5	8.7	34.8

TABLE 3.6 DR GENE FREQUENCIES IN CAUCASOID SLE AND CTD PATIENTS
COMPARED WITH CONTROLS.

DNA-DR TYPE	FREQUENCY											
	CONTROLS		SLE		PSS/CREST		SJOGREN'S		PM/DM		MCTD/UNCTD	
	N	%	N	%	N	%	N	%	N	%	N	%
DR1	8	5.8	15	9.1	9	16.1 ^a	0		3	10.0	5	13.2
DR2 (Dw2)	22	15.9	23	14.0	5	8.9	4	28.6	4	13.3	5	13.2
(Dw12)	2	1.4	0		0		0		0		0	
(DwKAS011)	0		1	0.6	1	1.8	1	7.1	0		0	
DR3	18	13.0	39	23.8 [*]	4	7.1	6	42.9 ^c	8	26.7	4	10.5
DR4	32	23.2	25	15.2	7	12.5	1	7.1	6	20.0	7	18.4
DR5	11	8.0	17	10.4	17	30.4 ^b	0		2	6.7	4	10.5
DRw13	14	10.1	15	9.1	4	7.1	0		0	^d	4	10.5
DRw14	7	5.1	4	2.4	1	1.8	0		0		4	10.5
DR7	19	13.8	24	14.6	4	7.1	1	7.1	5	16.7	3	7.9
DRw8	3	2.2	1	0.6	2	3.6	1	7.1	2	6.7	0	
DRw9	1	0.7	0		2	3.6	0		0		1	2.6
DRw10	1	0.7	0		0		0		0		1	2.6
Number Tested	69		82		28		7		15		19	

* $\chi^2_1=4.6$, $p<.05$

a $\chi^2_1=5.3$, $p<.05$

b $\chi^2_1=16.0$, $p<.001$

c $\chi^2_1=8.5$, $p<.005$

d $\chi^2_1=5.2$, $p<.05$

DRw13 at the 1% level. Some errors in antigen identification did occur, as shown in Table 3.5, but no systematic error emerged.

3.3.3 HLA-DR IN THE CAUCASOID SLE PATIENTS.

The frequencies of DNA-DR types in SLE compared with controls are shown in Table 3.6. The frequencies are markedly similar between SLE patients and controls, apart from DR3 which is increased in the SLE patients. This is statistically significant with a χ^2_1 value of 4.6 ($p < .05$) and relative risk of 1.5. The chi-square value has not been corrected for the number of antigens tested due to a priori hypothesis (Arnett et al. 1984) of an increase in DR3 antigen frequency in SLE.

3.3.4 HLA-DR IN CONNECTIVE TISSUE DISEASES.

HLA-DR distributions for the CTD patients are also shown in Table 3.6. A significant increase in DR5 compared to controls was seen in the PSS/CREST group ($\chi^2_1=16.2$, $p<.001$), which was also seen when both patient groups were analysed individually, with $\chi^2_1=11.1$ ($p<.001$) in the 13 scleroderma patients and $\chi^2_1=9.4$ ($p<.005$) in the 15 CREST patients. DR1 was also significantly increased in the PSS/CREST group ($\chi^2_1=5.3$, $p<.05$). The Sjogren's syndrome patients had a significant increase in HLA-DR3 ($\chi^2_1=8.5$, $p<.005$), but only 7 patients with primary Sjogren's syndrome were examined. The only other significant change in DR distributions in the CTD patients was a decrease in DRw6 in the Pm/Dm disease group ($\chi^2_1=5.2$, $p<.05$), with none of the fifteen patients positive for either of the DRw6 splits, DRw13 or DRw14.

TABLE 3.7 FREQUENCIES OF DNA-DQ TYPES IN CAUCASOID SLE AND CTD PATIENTS COMPARED WITH CONTROLS.

DQ TYPE	FREQUENCY											
	CONTROLS		SLE		PSS/CREST		SJOGREN'S		PM/DM		MCTD/UNCSTD	
	N	%	N	%	N	%	N	%	N	%	N	%
DQw1	54	39.1	58	35.4	20	35.7	5	35.7	7	23.3	19	50.0
DQw2	35	25.4	57	34.8	8	14.3	6	42.9	13 ^a	43.3	7	18.4
DQw3 (TA10+) (TA10-)	16	11.6	23	14.0	19	33.9 ^b	1	7.1	3	10.0	8	21.1
	33	23.9	26	15.9	9	16.1	2	14.3	7	23.3	4	10.5
Number Tested	69		82		28		7		15		19	

a $\chi^2_1=3.9$, $p<.05$

b $\chi^2_1=13.4$, $p<.001$

3.3.5 HLA-DQ IN THE CAUCASOID SLE AND CTD PATIENTS.

The SLE and CTD patients were examined for HLA-DQ types which were assigned according to linkage disequilibrium relationships with HLA-DR. As shown in Table 3.7, the SLE patients showed no significant differences compared with the controls in DQ distributions, with the increased frequency of DQw2 in the SLE disease group only approaching significance ($\chi^2_1=3.1$, $p>.05$)

DQw2 showed an increased frequency in the Pm/Dm patients ($\chi^2_1=3.9$, $p<.05$), but this is not significant when corrected for the number of antigens tested for. The PSS/CREST patients had an increase in DQw3/TA10+ ($\chi^2_1=13.4$, $p<.001$), but when corrected for the number of antigens tested, the χ^2 value only approached significance ($\chi^2_1=3.3$, $p>.05$).

3.3.6 DQ β /BamHI RFLPs IN THE SLE AND CTD PATIENTS.

The DQ β /BamHI RFLPs show associations with serological DR specificities (Owerbach et al. 1983) and can be used as an adjunct to the TaqI/DR β , /DQ α , and /DQ β RFLPs in DNA-DR typing. HLA-DR4/DQw3 cells can also be subdivided according to sizes of DQ β /BamHI fragments, such that TA10 positive cells have a 3.7kb fragment and TA10-negative cells have instead a 12kb fragment (Kim et al. 1985). There was a similar level of DR4-positive, TA10-positive or DR4-positive, TA10-negative cells in the SLE and CTD patients as in the controls. None of the other DQ β /BamHI RFLPs showed an increase in any of the SLE or CTD patients with the RFLPs correlating with expected DR types.

3.3.7 HLA-DR α /BglIII RFLPs IN SLE AND THE CTD PATIENTS.

As described by Stetler et al. (1982), DNA digested with BglIII gives four RFLPs, 4.5kb, 4.4kb, 3.9kb and 0.7kb in size, with the 3.9kb and 0.7kb fragments invariably occurring together. These DR types show linkage with HLA haplotypes (Serjeantson et al. 1986). The 4.5kb fragment occurs on the B18.DR3 haplotype while the 4.4kb RFLP is seen in B8.DR3 positive cells as well as DRw13 (Dw18) positive cells. The 3.9kb and 0.7kb fragments are associated with DR1, DRw10 and Bw57.DR7 positive haplotypes. These same associations are seen in the SLE and CTD patients studied here with no unexpected occurrences. No other DR α /BglIII RFLPs were seen in the SLE or CTD patients.

3.3.8 HLA-DP IN SLE AND THE CONNECTIVE TISSUE DISEASES.

The DP β gene in SLE and CTD patients was examined by RFLP analysis with the restriction enzymes BglIII and EcoRV. Two allelic fragments are observed with BglIII, approximately 20kb and 2.8kb in size, (Stetler et al. 1982), while EcoRV also gives two polymorphic fragments about 12kb and 8kb long, (Erlich and Stetler 1984). The DP β /BglIII phenotype and allele frequencies are shown in Table 3.8. No significant differences were seen between the controls and SLE or CTD patients in phenotype or allele frequencies and all phenotype distributions were in Hardy-Weinberg equilibrium. DP β /EcoRV allele frequencies and phenotype distributions in the controls and patients are given in Table 3.9. The SLE and CTD patients did not differ from the controls in frequencies of the DP β /EcoRV RFLPs. A recent report (Hyldig-Hielsen et al. 1987) shows that these DP RFLPs correlate with functional DP epitopes, with the

TABLE 3.8 DPβ/BglII POLYMORPHISMS IN CAUCASOID SLE AND CTD PATIENTS.

DPβ PHENOTYPE	CONTROLS		SLE		CREST/PSS		SJOGREN'S		PM/DM		MCTD/UNCTD	
	N	%	N	%	N	%	N	%	N	%	N	%
DPβ/BglII												
20kb/20kb	3	9.1	8	10.0	4	14.3	2	28.6	3	21.4	2	10.5
20kb/2.8kb	18	54.5	41	51.2	14	50.0	3	42.9	5	35.7	5	26.3
2.8kb/2.8kb	12	36.4	31	38.8	10	35.7	2	28.6	6	42.9	12	63.2
Allele frequency												
20kb	0.363		0.356		0.393		0.500		0.393		0.281	
2.8kb	0.636		0.644		0.607		0.500		0.607		0.719	
Total tested	33		80		28		7		14		19	

TABLE 3.9 DPβ/EcoRV POLYMORPHISMS IN CAUCASOID SLE AND CTD PATIENTS.

DPβ PHENOTYPE	CONTROLS		SLE		CREST/PSS		SJOGREN'S		PM/DM		MCTD/UNCTD	
	N	%	N	%	N	%	N	%	N	%	N	%
DPβ/EcoRV												
12kb/12kb	3	8.6	8	10.1	4	13.3	2	28.6	3	20.0	2	10.5
12kb/8kb	17	48.6	40	50.6	12	40.0	2	28.6	7	46.7	5	26.3
8kb/8kb	15	42.9	31	39.2	14	46.7	3	42.9	5	33.3	12	63.2
Allele frequency												
12kb	0.329		0.354		0.333		0.429		0.433		0.237	
8kb	0.671		0.646		0.667		0.571		0.567		0.763	
Total tested	35		79		30		7		15		19	

BglII/2.8kb and EcoRV/8kb fragments found in DPw2 and DPw4 cells, and the BglII/19kb and EcoRV/12kb fragments seen in DPw1, w3, w5, and w6 positive cells.

3.3.9 HLA-DR AND HLA-DQ IN JAPANESE PATIENTS WITH SLE.

A significant number of the Japanese SLE patients could not be DR typed serologically and DNA-DR typing proved very useful as it had been in Caucasoids. DR β /TaqI RFLPs in Japanese were the same as those seen in Caucasoids, with the exception of one DR5 associated RFLP and one rare DR4 variant pattern. The DR5 pattern occurs in Micronesians from Nauru and has been designated DR5*Nauru (Kohonen-Corish and Serjeantson 1986b). Instead of the usual 12kb and 6.0kb DR β /TaqI fragments seen in most DR5 positive Caucasoids, DR5*Nauru has a 10kb and a 4.4kb fragment. While DR5 in Caucasoids is associated with a 5.0kb DQ α /TaqI fragment, DR5*Nauru in the Japanese is associated with either a 5.0kb or 6.2kb fragment. The DQ α and DQ β RFLP associations with other DR types were essentially the same in the Japanese as Caucasoids except for DRw8 which usually had a 6.8kb DQ α /TaqI fragment rather than the 6.2kb fragment which is seen in Caucasoids. The DR and DQ RFLP patterns in Japanese compared with other populations in Asia-Oceania have been reported (Kohonen-Corish et al. 1988).

Table 3.10 gives DNA-DR frequencies in the Japanese SLE patients and controls. DR3 and DR7 were rare in the Japanese which is compatible with serological studies in this population. No significant differences were seen in the DR distributions between the SLE patients and controls with the increase in DR2 only approaching significance ($\chi^2_1=3.0$, $p>.05$). However, when DR2 was subdivided into RFLP types corresponding to HLA-D

TABLE 3.10 DNA-DR FREQUENCIES IN JAPANESE SLE PATIENTS
AND CONTROLS.

DNA-DR TYPE	FREQUENCY			
	CONTROLS (n=39)		SLE (n=40)	
	N	%	N	%
DR1	7	9.0	3	3.8
DR2 (Dw2)	6	7.7	16*	20.0
(Dw12)	7	9.0	6	7.5
(KAS011)	2	2.6	3	3.8
DR3	1	1.3	2	2.5
DR4	16	20.5	14	17.5
DR5	3	3.8	4	5.0
DRw13	6	7.7	3	3.8
DRw14	3	3.8	3	3.8
DR7	0		0	
DRw8	11	14.1	13	16.3
DRw9	15	19.2	13	16.3
DRw10	1	1.3	0	

* $\chi^2_1 = 5.0$, $p < .05$

specificities Dw2, Dw12 and Dw"KAS011", Dw2 was significantly increased ($\chi^2_1=5.0$, $p<.05$) in patients compared with controls.

HLA-DQ distributions were very similar between the Japanese SLE patients and controls, with DQw1 present in 41.0% of controls and 42.5% of patients, DQw2 seen in only 1.3% of controls and 2.5% of patients, and DQw3 present in 57.7% of controls and 55.0% of the SLE patients. Only one TA10-positive individual (an SLE patient) was seen in the Japanese studied here.

3.4 DISCUSSION.

3.4.1 DNA-DR TYPING IN SLE.

This study demonstrates an important application of DNA-DR typing techniques in an HLA associated disease where HLA-DR phenotypes cannot always be assigned using conventional tissue typing procedures. In SLE, investigations of HLA-D specificities using mixed lymphocyte cultures have proved technically unsuccessful (Arnett et al. 1984) due to poor viability of patient lymphocytes. Serological HLA-DR typing is similarly difficult in SLE patients. The situation is further exacerbated in patients undergoing corticosteroid therapy, due to decreased antigenic expression (Hokland et al. 1981; Snyder and Unanue 1982). In a study of white SLE patients (Gladman et al. 1979), one fifth could not be HLA-DR typed and in the current series, HLA-DR antigen assignment was not possible in one third of the SLE patients. This high failure rate has the potential to introduce bias into studies of HLA-DR antigens at risk for SLE, and there is also the potential for a systematic error in incorrect assignment of HLA-DR antigens. In a

series of SLE patients described by Ahearn et al. 1982, HLA-DR determinations were performed in duplicate and an unspecified proportion of patients was necessarily recalled when duplicated DR typings were discrepant.

In the current study, HLA-DR antigen assignment on serological results was attempted in 46 patients and 43% of the 92 antigens were either not detected or were designated incorrectly when compared with the DNA-DR typing results. This serious error rate can be compared with that in a healthy control series typed with the same HLA-DR antisera, where only 1% of the DR antigens were assigned incorrectly.

Given the high error rate in HLA-DR typing in SLE patients, the question remains as to whether the apparently conflicting results in the literature regarding the correlation of DR2 (Reinertsen et al. 1978; Reveille et al. 1985) and DR3 (Scherak et al. 1979; Celada et al. 1979) with SLE can be attributed to technical errors. In the current series, HLA-DR2 was correctly identified more often than any other antigen except DR3 and significantly more so than DRw13 and DR5; that is, the frequency of DR2 was overestimated when using serological typings. In earlier studies, this problem may have been exacerbated by mistyping. The definition of DRw6 has been difficult even in healthy individuals (Schreuder et al. 1984) and was first described in 1977 during the Seventh International Histocompatibility Workshop (Bodmer et al. 1978) using multispecific sera, HLA-DR2 + w6 and HLA-DR3 + w6. In the Eighth Histocompatibility Workshop in 1980, again, no monospecific sera could be defined and DRw6 was defined by DR3 + w6 sera. Thus, in DR3 positive individuals, HLA-DRw6 has proved particularly difficult to assign, in this as well as in earlier studies.

Technical problems are not of course the only factors contributing

to the conflicting reports regarding the association of DR2 or DR3 with SLE. If HLA-DR3 is not the primary susceptibility locus but is linked on chromosome 6 to the disease promoting genes, then different linkage disequilibrium relationships in different populations could account for the conflicting findings. Also, since SLE is a heterogeneous disorder, lack of clinical uniformity between different studies could account for discrepant reports in DR antigen frequencies. For instance, it has been claimed that the haplotype HLA-A1.B8.DR3 relates to more severe disease with higher titres of anti-DNA antibodies and HLA-B7.DR2 with milder disease (Christiansen et al. 1983; Griffing et al. 1980). However, in this study where unequivocal HLA-DR typings were available for all patients tested, HLA-DR3 was clearly and significantly associated with an increased risk for SLE and HLA-DR2 was not.

3.4.2 HLA-DQ IN SLE.

The increase in DQw2 frequency in the Caucasoid SLE patients is a consequence of the linkage disequilibrium between DQw2 and DR3. DQw2 RFLPs, when in linkage with DR7 did not increase in frequency in SLE patients. Ahearn et al. (1982) have reported an increase in DQw1 in SLE, but in their study DR2 was increased, so linkage disequilibrium may again be responsible. As the authors noted, the increase in DR2 was more significant than the increase in DQw1. It was also reported (Ahearn et al. 1982) that DQw1 positive patients had an increase in nRNP while those with DQw2 had an increase in anti-Ro antibodies, so it is possible that DQ antigens are segregating with different clinical subsets of SLE. This correlation between HLA class II RFLPs and clinical subsets of SLE is investigated further in Chapter 6.

3.4.3 HLA-DR AND HLA-DQ IN THE CONNECTIVE TISSUE DISEASES.

HLA-DR5 was increased in the CREST and PSS patients studied here which is in agreement with other studies of these diseases (Gladman et al. 1981; Briggs et al. 1986). DR1 was also increased in the CREST/PSS patients. Whether these are the primary susceptibility genes or are linked to the disease promoting locus cannot be determined and comparisons between different ethnic groups would be of value, as was shown here for SLE. PSS has shown associations with null genes at the complement component C4 loci and DR1 is linked to C4B*Q0 on certain haplotypes. DR5, however, is not linked to genes encoding complement deficiencies.

While an association was seen between DQw3 and the connective tissue diseases CREST and scleroderma, this may be because of linkage disequilibrium with the DR antigens, in particular with DR5 which was significantly increased.

Earlier studies have shown DR3 and DQw2 to be increased in patients with primary Sjogren's syndrome (Wilson et al. 1984), and with the small numbers of patients studied here, the same increases were seen. The association of DQw2 may be secondary to the DR3 increase due to linkage disequilibrium.

The only other connective tissue disease with a significant change in DR or DQ distributions was the polymyositis/dermatomyositis group where none of the 15 patients were positive for DRw13 or DRw14. This could have been an effect of the small patient numbers. DR3 was increased in one study of 12 polymyositis patients (Kirsch et al. 1980) and a similar trend was seen in the current study.

3.4.4 HLA-DP RFLPs IN THE SLE AND CTD PATIENTS.

From the RFLPs examined in the DP genes, using BglII and EcoRV, there does not appear to be any association with SLE or the connective tissue diseases studied here. With the recent advances in DNA-DP typing, it should now be possible to correlate functional variation in HLA-DP with SLE and the CTDs.

3.4.5 DR TYPING IN JAPANESE SLE PATIENTS.

The Japanese SLE patients here showed a significant increase in DR2(Dw2) RFLPs. This is in accord with the previous findings of an increase in DR2 (Hashimoto et al. 1985). There was no decrease in DR4 as observed by Kameda et al. (1982) in Japanese SLE patients. Since the increased risk for SLE is associated with DR2(Dw2) in Japanese and with DR3 in Caucasoids, it would seem that HLA-DR is not the primary disease-promoting gene but is in linkage disequilibrium with a disease susceptibility locus.

3.5 CONCLUSION.

In this study, DNA-DR typing has shown errors in serological DR typing of Caucasoid SLE patients which is partially systematic. This could cause inflation in the estimate of DR2 antigen frequencies. The practical application of recombinant DNA techniques in HLA-DR typing has shown HLA-DR3, but not HLA-DR2, to be significantly associated with an increased risk for SLE in Caucasoids. No association was seen between SLE and the DQ antigens, nor with DP RFLPs in the Caucasoid patients.

DNA-DR typing has also been useful in the study of Japanese patients with SLE, revealing an increase in Dw2 in the patients. DQ does not appear to be associated with SLE in the Japanese.

An increase in DR5 in the CREST/PSS patients with DNA-DR typing supports earlier serological observations, but the patient numbers in the other CTDs examined are too small to make definite conclusions. No strong associations were seen between the connective tissue diseases and DQ or DP genes.

CHAPTER FOUR

COMPLEMENT AND SLE

4.1 INTRODUCTION.

Complete deficiencies of complement components C4 and C2 have clearly been associated with SLE in several studies (reviewed by Agnello 1986; Atkinson 1986). Homozygous C2 deficiency is the most common complete deficiency of complement and was the first complement deficiency associated with SLE. Of the published cases of homozygous C2 deficiency, about 75% of the individuals suffer from SLE or an immune complex mediated illness such as glomerulonephritis. Complete deficiency of C4 is rare as this requires four null or defective genes, but of the 15 reported individuals with complete C4 deficiency, eight had SLE and another three had an SLE like disease. Partial C4 deficiency has also been implicated in SLE in some studies (Fielder et al. 1983; Reveille et al. 1985), as has partial deficiency of C2 (Glass et al. 1976), although one study (Christiansen et al. 1983) reported no increase in C2 deficiency in their patient series. However, because of the linkage disequilibrium between the MHC class I and II genes, the importance of partial C2 and C4 deficiencies in the pathogenesis of SLE has been difficult to determine.

While complete deficiency of C4 or C2 could understandably lead to an immune complex disease, the mechanism of partial deficiency is less clear when an individual may, for example, have three functional C4 alleles and only one null allele. The situation becomes more confusing when considering the many healthy people who have partial deficiencies of C2 or C4. In fact, the first reported case of complete C2 deficiency was seen in an immunologist who donated some blood for a complement fixing experiment which didn't work (Silverstein 1960).

It is now becoming evident that C4A and C4B alleles have different biological properties, which was initially seen when C4B alleles showed greater haemolytic activity than C4A alleles. This has become the basis for differentiating between overlapping C4A and C4B alleles when C4 allotyping (Awdeh and Alper 1980). C4B has now been shown to react more quickly than C4A with hydroxyl groups which are found in abundance on the surface of red blood cells (Isenman and Young 1984; Law et al. 1984). The relative importance of C4A and C4B null alleles in SLE is not clear with two previous studies of SLE and complement component C4 showing an increase of C4A and C4B in familial SLE (Fielder et al. 1983; Reveille et al. 1985), while another study by Christiansen et al. (1983) indicating that only C4A*Q0 was increased in lupus. A recent study of DR3-negative SLE patients has shown an increase in C4B*Q0 (Batchelor et al. 1987).

One problem with studying C4 deficiencies in SLE is that it is sometimes difficult to assign null alleles by gel electrophoresis results, as this is dependant on differences in staining intensity between the C4A and C4B alleles. This becomes impossible when an individual is both heterozygous C4A and C4B deficient, as the C4A:C4B density ratio remains 1:1. Examination of serum C4 levels to look for C4 deficiencies is not always reliable because of the wide range in different people (Schur 1986). To attempt to solve this problem, studies have been made at the genomic level to search for RFLPs that will detect C4 null alleles.

Several investigators have reported a TaqI RFLP with a 21-OH probe which detects null alleles on the two haplotypes A1.B8.DR3.C4A*Q0 and B18.DR3.C4B*Q0 (White et al. 1984; Carroll et al. 1985; Garlepp et al. 1986). A 3.2kb fragment is absent in individuals homozygous for these

haplotypes due to a large deletion encompassing either the C4A or C4B gene and the 21-OHA gene. However in individuals heterozygous for one of these haplotypes, differences in density between the 3.2kb band and other RFLPs are required to detect the null allele and this is not always a reliable method. One RFLP has been reported to identify individuals with a deleted C4A gene (Carroll et al. 1985) with a 15kb HindIII fragment lost and an 8.5kb fragment gained when the probe pAT-A (Belt et al. 1985) is used. However not all C4 deficiencies are believed to have arisen through deletions. Schneider et al. (1986) estimate that 50% of C4A or C4B null alleles are the result of large deletions in the C4 genes that also include part of one of the 21-OH genes which flank the C4 genes. The basis of the remaining 50% of null alleles is not known, and at the moment cannot be detected by RFLPs. One possibility put forward by Palsdottir et al. (1987) is that of gene conversion so that a chromosome could end up with two C4A or two C4B genes.

In addition to the problems of assigning C4 null genes, linkage disequilibrium within the MHC makes it difficult to determine what gene is the most important in contributing to SLE. For example, in Caucasoids, DR3 has been reported to be increased in SLE (Reinertsen et al. 1978; Reveille et al. 1985) as have C4A and C4B null alleles (Fielder et al. 1983; Reveille et al. 1985; Christiansen et al. 1983). However, C4A*Q0 is in linkage disequilibrium with the haplotype A1.B8.DR3, while C4B*Q0 is found on the B18.DR3 haplotype. Thus it has been difficult to distinguish between the class II and class III genes as the primary susceptibility gene in SLE.

One way of overcoming linkage disequilibrium effects is to examine SLE patients of different ethnicities. The class I and II HLA antigens

and class III complement allotypes vary in frequency between various ethnic groups, and also show different linkage disequilibrium relationships. For example, while DR3 is common in Caucasoids, it is rare in Japanese. Similarly, the haplotype A24.B52.DR2 is rare in Caucasoids but common in Japanese. Different ethnic groups can be examined to look for a common susceptibility locus which crosses ethnic boundaries. To avoid the problems of assigning C4 alleles, gene frequencies can be estimated by statistical techniques, for example by the maximum likelihood method, with iteration of the null allele frequencies to obtain the best fit to Hardy-Weinberg equilibrium of the observed phenotype distributions.

4.1.1 AIMS.

The contribution of deficiencies of the early complement components, C2 and C4, to the pathogenesis of SLE have been investigated by examining C4 allelic distributions in SLE patients in three distinct ethnic groups, Caucasoid, Chinese and Japanese, and by C2 functional assays in these three different populations. C2 phenotypes in Caucasoid SLE patients were also examined. C4 phenotyping, C2 functional assays and C2 phenotyping have also been done in some CTD groups, to look for any associations of these diseases with C2 and C4. The contribution of C4 to lupus is compared with that of HLA-DR.

4.2 MATERIALS AND METHODS.

4.2.1 PATIENTS AND CONTROLS.

Japanese and Chinese SLE patients were examined for HLA antigen distributions as part of the Third Asia Oceania Histocompatibility Workshop (Naito et al. 1986) as were healthy Japanese and Hong Kong Chinese. The Japanese patients were reviewed by Dr. S. Naito, and the Hong Kong Chinese by Dr. B. Hawkins. Plasma was stored at -70°C within a few hours of collection and forwarded in dry ice to Canberra. Caucasoid patients with SLE and the related diseases scleroderma, CREST, Sjogren's syndrome, polymyositis (Pm) and/or dermatomyositis (Dm), mixed connective tissue disease (MCTD) and undifferentiated connective tissue disease (UNCTD) have been described in Chapters 2 and 3. Sterile blood was transported overnight to Canberra where EDTA plasma was separated and stored at -70°C . Caucasoid controls came from the Canberra Blood Bank and EDTA plasma samples were stored at -70°C within a few hours of collection.

4.2.2 C4 TYPING.

C4 allotyping was performed on patients and controls following the method of Awdeh and Alper (1980) as described in Chapter 2. Haemolytic overlays were carried out when C4A and C4B alleles overlapped or when it was difficult to distinguish between C4A and C4B alleles. Caucasoid controls were typed by Mrs. P. Ranford while the Caucasoid patients and all the Japanese and Chinese were typed by myself. The C4 gels were read independently by at least one other person.

4.2.3 STATISTICAL ANALYSIS.

Differences in C4A and C4B phenotype distributions between patients and controls were tested by the chi-square statistic with continuity correction. If any number in a cell of a 2x2 contingency table fell below 5, the exact probability was calculated (Armitage 1971). Frequencies of the common haplotypes C4A*3.B*1 and C4A*4.B*2 were calculated according to Mittal et al. (1973) and the nonrandom association of C4A and C4B alleles was measured by the disequilibrium statistic delta. (Mittal et al. 1973). The significance of delta was tested by chi-square.

4.2.4 ESTIMATION OF NULL ALLELE FREQUENCIES.

Partial deficiency of either C4A or C4B can sometimes be inferred by differential density of C4A and C4B bands on an agarose gel, (for example Figure 4.1a, lanes 1 and 4), but an individual heterozygous for both null alleles cannot be detected by this technique as the C4A:C4B ratio densities remains 1:1 (Figure 4.1b, lane 3). The population frequencies can be determined however, by statistical techniques. Gene frequencies were estimated using the maximum likelihood method, with iteration of the null allele frequencies to obtain the best fit of observed phenotype distributions to Hardy-Weinberg equilibrium. Sjogren's syndrome and Pm/Dm could not be examined by this method because of small patient numbers.

4.2.5 LOGISTIC REGRESSION.

Logistic regression was used to compare the contribution of C4A or C4B deficiency with DR3 to lupus. The SPSS^x statistical package was used with the loglinear function (SPSS^x Users Guide 1986). Individuals with SLE were designated as either homozygous for C4A*Q0, heterozygous for C4A*Q0 (determined by density differences between C4A and C4B), heterozygous for two C4A alleles (definitely not C4A deficient) or undetermined (because the C4A:C4B density ratio was 1:1). C4B phenotypes were assigned in the same way. The Caucasoid SLE patients were compared with Caucasoid control haplotype data analysed in the Ninth International Histocompatibility Workshop (Baur et al. 1984). These haplotypes were obtained from family studies. The published haplotype frequencies were used to calculate the numbers of individuals with and without C4A*Q0 (both homozygous and heterozygous C4A*Q0), the number of those with and without DR3 (homozygous or heterozygous for DR3) and the joint occurrence of these alleles. Similarly, the C4B*Q0, DR3 data was calculated from the haplotype frequencies.

4.2.6 HYBRIDIZATIONS.

DNA from selected individuals was digested with TaqI and probed with a C4 cDNA probe following the methods described in Chapter 2. The 950bp probe pC4AL1 (Whitehead et al. 1984) was kindly provided by Dr. A.S. Whitehead.

4.2.7 C2 TYPING.

Caucasoid patients with SLE or a connective tissue disease were C2 typed by isoelectric focussing on polyacrylamide gels, following the method of Awdeh (1968), as described in Chapter 2. C2 gels were read independently by another person.

4.2.8 C2 FUNCTIONAL ASSAYS.

C2 functional assays were performed on Caucasoid, Japanese, and Chinese SLE patients, Caucasoid CTD patients and controls, according to Lachmann and Hobart (1978), as described in Chapter 2. The C2 assays of the patients were compared with two controls known to have 50% and 100% C2 levels, and were designated as having either 100% C2 (same amount of haemolysis as the 100% control), 50% C2 (same amount of haemolysis as the 50% control), 0% (no haemolysis at all) or 75% (between 50% and 100% haemolysis level). The C2 assay results were checked independently by another person.

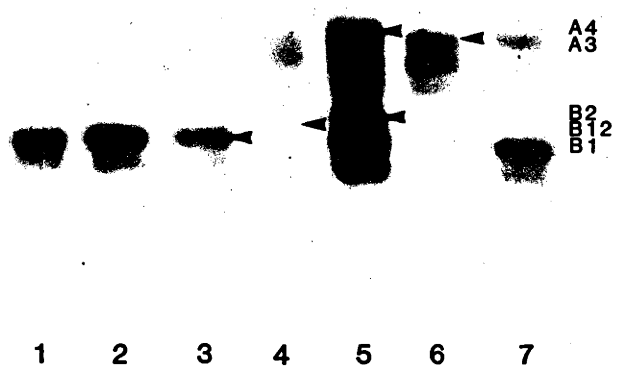
4.3 RESULTS.

4.3.1 C4 IN CAUCASOID, JAPANESE, AND CHINESE SLE PATIENTS AND IN CAUCASOID CTD PATIENTS.

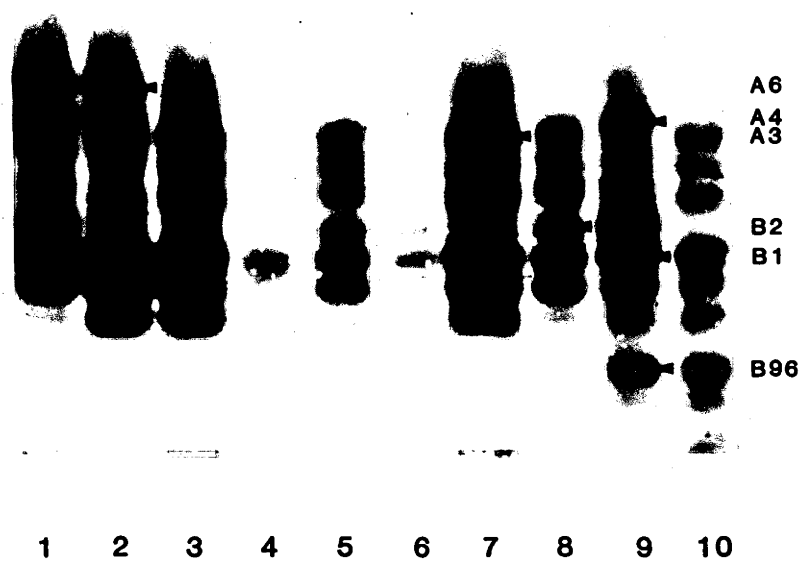
Figure 4.1 is a photo of a typical C4 gel stained with Coomassie Blue. Each gene product is represented by a set of three bands and phenotypes are indicated by the position of the leading band. C4A alleles are generally more acidic compared to C4B, but there is an

- FIGURE 4.1 a. Photograph of a C4 gel stained with Coomassie blue showing null alleles. Lane 1: C4A*3,Q0 C4B*1,1; Lane 2: C4A*Q0,Q0 C4B*1; Lane 3: C4A*3 C4B*1; Lane 4: C4A*3,3 C4B*12; Lane 5: C4A*4 C4B*1,2; Lane 6: C4A*3 C4B*Q0,Q0; Lane 7: C4A*3 C4B*1.
- b. Photograph of a C4 gel showing the slow C4B variant seen in Chinese. Lane 1: C4A*4,6 C4B*1,2; Lane 2: C4A*3,6 C4B*1,1; Lane 3: C4A*3 C4B*1; Lane 4: C4A*3 C4B*1; Lane 5: C4A*4 C4B*1,2; Lane 6: C4A*3 C4B*1,2,96; Lane 7: C4A*3 C4B*1; Lane 8: C4A*4 C4B*1,2; Lane 9: C4A*4 C4B*1,2,96; Lane 10: C4A*3 C4B*1,96.

a.



b.



overlapping zone and haemolytic assays are required to differentiate between C4A and C4B gene products when they lie in this area.

Table 4.1 shows the C4A gene frequency distributions in Caucasoid, Japanese and Chinese SLE patients and controls. C4A*3 was the most common allele in all populations with frequencies ranging between 45% in Japanese SLE patients to 69% in Caucasoid controls. The Japanese showed the least genetic variability, with C4A*5 and C4A*12 absent and very few instances of C4A*2 and C4A*6. Table 4.1 shows that although the C4A null gene (C4A*Q0) reached appreciable frequencies in all populations, there was a systematic two to three fold increase in frequency in each patient group with $p < .01$ in the Caucasoids ($X^2_1 = 13.5$) and Japanese ($X^2_1 = 13.7$), and $p < .05$ in the Chinese ($X^2_1 = 5.3$). There was a compensatory decrease in C4A*3 that reached statistical significance in the Japanese ($X^2_1 = 10.5$, $p < .01$) and Chinese ($X^2_1 = 9.5$, $p < .01$). No patient group showed an increase in the less haemolytically active C4A*6 allele compared with controls.

Table 4.2 shows C4B gene frequency distributions. In all populations the most common allele was C4B*1, although C4B*2 reached frequencies of 41% in Chinese controls. Once again, the Japanese showed the most restricted range of genetic variation. In the Japanese, C4B*3 and C4B*12 were absent and C4B*4 was rare. The frequency of C4B null alleles ranged from 13% in Chinese to 20% in Caucasoid controls, with no increase in SLE patients.

An unusual C4B allele was seen in the Chinese, the most cathodal or basic C4B allele yet described, (shown in Figure 4.1b, lanes 6, 9 and 10) (Dunckley and Hawkins 1986). The variant is functional as evidenced by the haemolytic overlay technique and has been designated C4B*96 according to guidelines devised by the IV International Workshop for

Table 4.1 C4A GENE FREQUENCIES IN CAUCASOID, JAPANESE, AND CHINESE SLE PATIENTS AND CONTROLS.

Allele	Caucasoid				Japanese				Chinese			
	SLE n=79	GF ^a	SE ^b	Controls n=197	GF	SE	SLE n=51	GF	SE	Controls n=50	GF	SE
C4A*2	0.032	0.014	0.040	0.010	0.000	0.010	0.000	0.010	0.010	0.069	0.021	0.040
C4A*3	0.564	0.050	0.685	0.032	0.458*	0.058	0.684	0.062	0.413*	0.046	0.590	0.050
C4A*4	0.061	0.019	0.069	0.013	0.185	0.040	0.175	0.039	0.194	0.034	0.162	0.031
C4A*5	0.000		0.003	0.003	0.000	0.000	0.000	0.000	0.000	0.007	0.007	0.007
C4A*6	0.032	0.014	0.031	0.009	0.010	0.010	0.010	0.010	0.013	0.094	0.013	0.009
C4A*12	0.000		0.005	0.004	0.000	0.000	0.000	0.000	0.007	0.007	0.000	0.000
C4A*Q0	0.311*	0.048	0.169	0.029	0.347*	0.058	0.121	0.056	0.304 [†]	0.046	0.188	0.046

a GF=Gene frequency b SE=Standard error
 + .01<p<.05 * p<.01

Table 4.2 C4B GENE FREQUENCIES IN CAUCASOID, JAPANESE, AND CHINESE SLE PATIENTS AND CONTROLS.

Caucasoid				Japanese				Chinese				
SLE n=79	Controls n=197	SLE n=51	Controls n=50	SLE n=75	Controls n=76							
Allele	GF ^a	SE ^b	GF	SE	GF	SE	GF	SE	GF	SE		
C4B*1	0.710	0.051	0.668	0.032	0.572	0.059	0.585	0.061	0.475	0.047	0.402	0.044
C4B*2	0.087	0.023	0.112	0.016	0.180	0.040	0.172	0.039	0.334	0.042	0.412	0.044
C4B*3	0.013	0.009	0.018	0.007	0.000		0.000		0.000		0.021	0.012
C4B*4	0.000		0.003	0.003	0.000		0.020	0.014	0.000		0.000	
C4B*5	0.000		0.005	0.004	0.106	0.031	0.031	0.017	0.007	0.007	0.026	0.013
C4B*12	0.006	0.006	0.000		0.000		0.000		0.000		0.000	
C4B*96	0.000		0.000		0.000		0.000		0.042	0.016	0.013	0.009
C4B*Q0	0.183	0.049	0.195	0.030	0.142	0.052	0.193	0.056	0.143	0.043	0.126	0.042

a GF=Gene frequency b SE=Standard error

Genetics of Complement (Mauff et al. 1983). C4B*96 was not described in the study of complement allotypes in Orientals in the IXth International Histocompatibility Workshop. C4B*96 was higher in frequency in the Cantonese SLE patients compared with normal controls with 7 of 77 (9.1%) of the SLE patients having the C4B*96 allele compared with 2 of 76 (2.6%) of controls ($0.05 < p < 0.1$). The C4B*96 allele appears to be on a chromosome with a duplicated C4B locus with all individuals positive for C4B*96 also C4B*1 positive, and in three of the nine occurrences of C4B*96, C4B*1 and C4B*2 alleles were both present. Significant linkage disequilibrium was seen in the Cantonese between C4B*96 and HLA-B13. Seven of nine (77.5%) were positive for C4B*96 and HLA-B13 ($\chi^2_1 = 22.0$, $p < 0.001$). C4B*96 was also seen in two Chinese SLE patients, now resident in Sydney, both of whom also carried C4B*1 and C4B*2 and were HLA-B13 positive.

Table 4.3 shows C4A gene frequency distributions in the connective tissue disease patients compared with controls. C4A*3 was the most common allele in the patient groups studied followed by C4A*4. The C4A*Q0 allele was increased in the MCTD/UNCTD group ($\chi^2_1 = 6.4$, $p < .05$) and was due to an increase of apparent C4A,C4B homozygosity in the MCTD/UNCTD patients (there were no C4A or C4B null homozygotes). Although all patients appeared to be homozygous at the C4A locus, some could have been carriers for both C4A*Q0 and C4B*Q0 alleles.

Table 4.4 shows C4B gene frequencies in the CTD patients and controls. A similar distribution is seen between the patient groups and the controls with C4B*1 and C4B*2 comprising 70 to 77% of the C4B alleles in the CREST/PSS and MCTD/UNCTD disease groups. Apart from the C4B*Q0 allele, the other C4B alleles were rare in these disease groups.

Table 4.3 C4A GENE FREQUENCIES IN CONNECTIVE
TISSUE DISEASE PATIENTS AND CONTROLS.

	Controls		PSS/CREST		MCTD/UNCTD	
	n=197		n=33		n=20	
Allele	GF ^a	SE ^b	GF	SE	GF	SE
C4A*2	0.040	0.010	0.000		0.026	0.025
C4A*3	0.685	0.032	0.657	0.078	0.536	0.098
C4A*4	0.069	0.013	0.100	0.038	0.083	0.044
C4A*5	0.003	0.003	0.000		0.000	
C4A*6	0.031	0.009	0.046	0.026	0.000	
C4A*12	0.005	0.004	0.000		0.000	
C4A*Q0	0.169	0.029	0.197	0.073	0.355*	0.096

a GF=Gene frequency b SE=Standard error

* p<.01

Table 4.4 C4B GENE FREQUENCIES IN CONNECTIVE
TISSUE DISEASE PATIENTS AND CONTROLS.

	Controls		PSS/CREST		MCTD/UNCTD	
	n=197		n=33		n=20	
Allele	GF ^a	SE ^b	GF	SE	GF	SE
C4B*1	0.668	0.032	0.630	0.078	0.638	0.010
C4B*2	0.112	0.016	0.144	0.045	0.079	0.043
C4B*3	0.018	0.007	0.015	0.015	0.027	0.026
C4B*4	0.003	0.003	0.000		0.000	
C4B*5	0.005	0.004	0.000		0.027	0.026
C4B*Q0	0.195	0.030	0.211	0.073	0.205	0.092

a GF=Gene frequency b SE=Standard error

Table 4.5 gives C4A,C4B and C4A.C4B phenotype distributions in Caucasoid SLE patients and controls. Individuals typing as apparent homozygotes for C4A or C4B alleles may in fact be carriers of null alleles for C4A or C4B, so phenotypes such as C4B 1-1 and C4B 1-Q0 are pooled. There is an increased frequency of C4A*Q0 homozygosity in SLE patients compared with controls ($\chi^2_1=5.1$, $p<.05$), with the C4A*Q0 allele occurring in conjunction with C4B*1, C4B*2 or C4B*3. There is no increase in C4B*Q0 homozygosity in patients. The frequency of C4A*3-4 is decreased in SLE patients although not significantly ($\chi^2_1=3.7$, $p(\text{corr})=.06$). This is accompanied by a small increase in C4A*4-4,4-Q0 ($\chi^2_1=2.6$, $p=.1$). [$p(\text{corr})=p(\text{corrected})$].

The distribution of C4 phenotypes in Japanese SLE patients and controls is given in Table 4.6. There is a significant increase in the frequency of C4A null homozygosity in patients ($\chi^2_1=4.3$, $p<.05$) but no increase in C4B null homozygosity. The C4A*Q0 allele occurs in conjunction with C4B*1, C4B*2 or C4B*5 in Japanese as shown in Table 4.6, so no secondary distortion in C4B phenotypes was evident in the SLE patients.

Table 4.7 shows the C4A and C4B phenotype distributions in the Chinese SLE patients and controls. The most common phenotype is C4A*3-3,3-Q0 with the C4A*3 allele linked with several different C4B alleles. The frequency of C4A*3-4 is decreased in SLE patients, although not significantly ($\chi^2_1=3.4$, $p(\text{corr})=.06$). This would be compensated for if some of the apparent C4A.C4B homozygotes were carriers for the C4A and C4B null alleles.

Linkage relationships between C4A*3 and C4B*1 and between C4A*4 and C4B*2 in the three populations for patient and control groups are shown in Table 4.8. C4A*4 and C4B*2 are in significant linkage disequilibrium

Table 4.5 DISTRIBUTION OF C4A AND C4B PHENOTYPES IN CAUCASOID SLE PATIENTS AND CONTROLS.

C4B PHENOTYPE																
1-1,1-Q0		1-2		1-3		2-2,2-Q0		3-3,3-Q0		Others		Q0-Q0		TOTAL		
SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	
PHENOTYPE	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
2-2,2-Q0	1	1.3	4	2.0	0	0	0	0	0	0	0	0	0	1	1.3	5 2.5
2-3	2	2.6	5	2.5	0	0	1	1.3	0	0	0	0	0	1	0.5	3 3.6
3-3,3-Q0	48	63.2	117	59.4	1	1.3	2	1.0	1	1.3	3	1.5	1	1.3	5 2.5	53 69.7 72.6
3-4	0	0	2	1.0	0	0	5	2.5	0	0	1	1.3	0	0	1	1.3 17 8.6
3-6	2	2.6	6	3.0	0	0	0	0	0	0	0	0	1	1.3	0	3 3.0
4-4,4-Q0	1	1.3	0	0	4	5.3	5	2.5	0	0	2	1.6	1	0.5	0	7 9.2 7 3.6
6-6,6-Q0	1	1.3	1	0.5	0	0	0	0	0	0	0	0	0	0	0	1 1.3 2 1.0
Others	1	1.3	1	0.5	0	3	1.5	0	0	0	0	0	0	2	1.0	1 1.3 7 3.6
Q0-Q0	3	3.9	2	1.0	3	3.9	0	0	1	0.5	0	0	0	0	6*	7.9 3 1.5
TOTAL	59	77.6	138	70.1	8	10.5	33	16.8	1	1.3	4	2.0	4	5.3	9 4.6	1 1.3 3 1.5 2 2.6 8 4.0 76 100.0 197 100.0

* .01<p<.05

Table 4.6 DISTRIBUTION OF C4A AND C4B PHENOTYPES IN JAPANESE SLE PATIENTS AND CONTROLS.

C4B PHENOTYPE																
1-1,1-Q0		1-2		1-5		2-2,2-Q0		5-5,5-Q0		Others		Q0-Q0		Total		
SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	
PHENOTYPE	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %
C4A																
3-3,3-Q0	20 39.2	28 56.0	1 2.0	0	3 5.9	2 4.0	0	2 3.9	1 2.0	1 2.0	1 2.0	0	1 2.0	27 52.9	33 66.0	
3-4	1 2.0	1 2.0	6 11.8	4 8.0	0	1 2.0	3 6.0	0	0	1 2.0	0	0	8 15.7	9 18.0		
4-4,4-Q0	2 3.9	0	0	3 6.0	2 3.9	0	5 9.8	3 6.0	0	0	0	0	9 17.6	6 12.0		
Others	1 2.0	0	0	1 2.0	0	0	1 2.0	0	0	0	0	0	1 2.0	2 4.0		
Q0-Q0	2 3.9	0	2 3.9	0	1 2.0	0	0	1 2.0	0	0	0	0	6* 11.8	0		
Total	26 51.0	29 58.0	9 17.6	8 16.0	6 11.8	2 4.0	6 11.8	7 14.0	3 5.9	1 2.0	1 2.0	2 4.0	1 2.0	51 100.0	50 100.0	

*.01<p<.05

Table 4.7 DISTRIBUTION OF C4A AND C4B PHENOTYPES IN CHINESE SLE PATIENTS AND CONTROLS.

C4B PHENOTYPE																													
1-1,1-Q0		1-2		1-5		2-2,2-Q0		1-1,96		2-1,96		Others		Q0-Q0		Total													
SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON	SLE	CON												
C4A																													
PHENOTYPE	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%											
2-2,2-Q0	3	4.0	0	0	1	1.3	0	0	0	0	0	0	0	0	0	3	4.0	1	1.3										
2-3	6	8.0	2	2.6	1	1.3	2	2.6	0	0	0	0	0	0	0	7	9.3	4	5.3										
3-3,3-Q0	12	16.0	14	18.4	12	16.0	14	18.4	4	5.3	6	8.0	10	13.2	5	6.7	2	2.6	1	1.3	37	49.3	47	61.8					
3-4	0	1	1.3	1	1.3	5	6.6	0	0	1	1.3	3	3.9	0	0	0	0	0	0	0	2	2.7	9*	11.8					
4-4,4-Q0	2	2.7	1	1.3	10	13.3	4	5.3	0	0	8	10.7	7	9.2	1	1.3	0	0	0	0	21	28.0	12	15.8					
Others	2	2.7	0	1	1.3	0	0	0	0	2	2.6	0	0	1	1.3	0	0	1	1.3	0	0	3	4.0	3	3.9				
Q0-Q0	1	1.3	0	1	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2.7	0	0					
Total	26	34.7	18	23.7	26	34.7	26	34.2	0	4	5.3	15	20.0	22	28.9	6	8.0	2	2.6	1	1.3	3	3.9	1	1.3	75	100.0	76	100.0

*p<.05

Table 4.8 C4A,C4B LINKAGE RELATIONSHIPS IN SLE PATIENTS
AND CONTROLS IN THREE ETHNIC GROUPS.

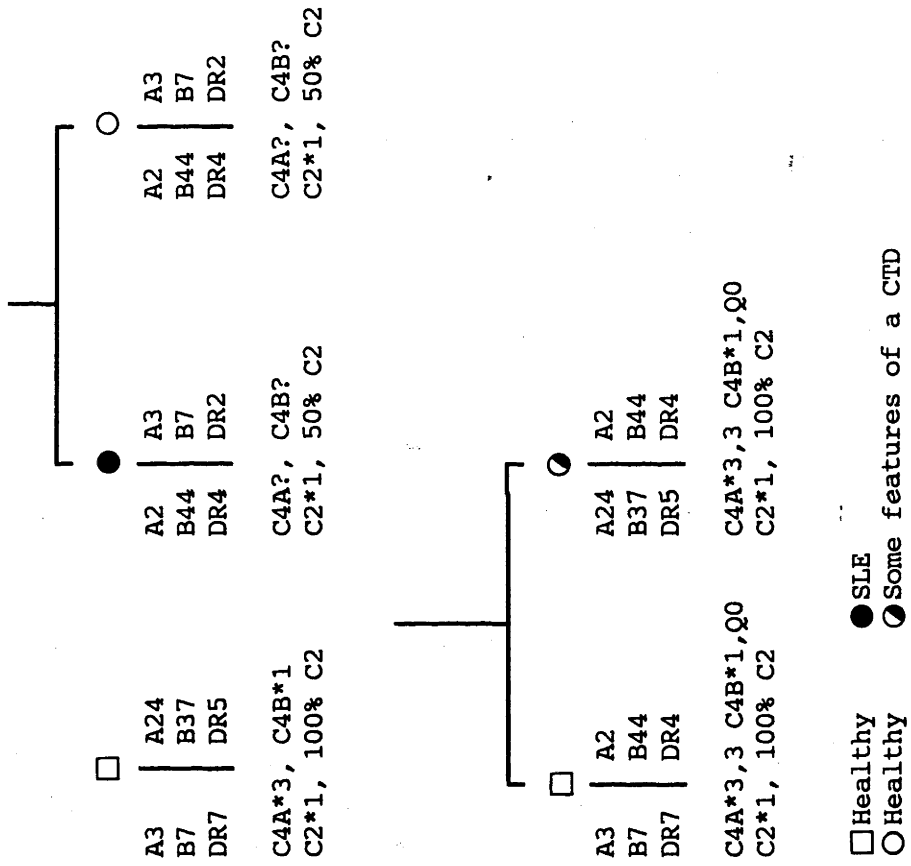
		C4 HAPLOTYPE			
		C4A*3.B*1		C4A*4.B*2	
ETHNICITY	DISEASE STATUS	HF ^a	Δ^b	HF	Δ
Caucasoid	SLE	0.379	0.013	0.040	0.036**
	Control	0.459	0.026	0.060	0.052**
Japanese	SLE	0.340	0.095*	0.123	0.094**
	Control	0.414	0.095*	0.139	0.112**
Chinese	SLE	0.229	0.031	0.147	0.091**
	Control	0.319	0.094**	0.126	0.064**

a HF= haplotype frequency

b Δ = disequilibrium statistic delta

* .01<p<.05 ** p<.01

FIGURE 4.2 FAMILY PEDIGREE OF AN SLE PATIENT WITH NO DETECTABLE C4.



in all groups while C4A*3 and C4B*1 are linked only in Chinese controls and in the Japanese groups. The linkage disequilibrium value was significant for the haplotype C4A*3.B*2 in the Chinese patients ($\chi^2_1=3.9$, $p<.05$) and controls ($\chi^2_1=5.2$, $p<.05$) but was not a common haplotype in the Caucasoids or Japanese.

The numbers of patients in the CTD groups were too small to look at C4A,C4B phenotype distributions however the Pm/Dm patients showed an increase in C4A*4,B*2 with 5/19 (26%) positive for these two alleles compared to 21/197 (11%) of the controls ($\chi^2_1=2.7$, $p(\text{corr})=.1$). Two of the five Pm/Dm patients with C4A*4 and C4B*2 were apparent homozygotes for these two alleles compared to 1/21 controls ($\chi^2_1=4.9$, $p<.05$).

One family was studied with twin sisters, one with lupus and the other healthy. The two children and husband of the SLE twin were also available for study (Figure 4.2). Both twins were identical at the HLA class I and II loci and both had undetectable levels of C4. However, from the children it was clear that the genes for C4 were present in the SLE twin. DNA from the twins was also examined with a C4 cDNA probe and this confirmed that the C4 genes were present in both twins. The twins also presented with 50% C2 levels so it appears that another factor such as C1 esterase inhibitor deficiency is causing the low C4 and C2 levels seen in these twin sisters.

4.3.2 C4A DEFICIENCY VERSUS HLA-DR3 IN SLE.

The relative contribution of DR3 and C4A deficiency to SLE in Caucasoids was analysed by logistic regression. Both complete and partial C4A deficiencies were compared with DR3. Patients and controls were divided into the four groups shown in Table 4.9 as described in the

Table 4.9 C4A*Q0 AND DR3 IN CAUCASOID SLE PATIENTS AND CONTROLS.

C4A TYPE	SLE		CONTROLS	
	DR3+	DR3-	DR3+	DR3-
Two C4A null alleles	6	0	5	2
One C4A null allele	16	8	56	51
No C4A null alleles	9	14	31	207
Undetermined	6	19	26	124
Total	37	41	118	384

Table 4.10 COMPLETE C4A DEFICIENCY VERSUS DR3.

VARIABLES IN MODEL	X ² (GOF)**	DF*	p
C4A*Q0-Q0 ⁺	4.6	2	0.098
C4A*Q0-Q0, DR3	4.3	1	0.038
DR3	14.6	2	0.001

* DF= degrees of freedom ** GOF= goodness of fit
+ C4A*Q0-Q0= complete C4A deficiency

Table 4.11 PARTIAL C4A DEFICIENCY VERSUS DR3.

VARIABLES IN MODEL	X ² (GOF)**	DF*	p
C4A*Q0-x ⁺	3.5	2	0.173
C4A*Q0-x, DR3	3.4	1	0.066
DR3	8.2	2	0.017

* DF= degrees of freedom ** GOF= goodness of fit
+ C4A*Q0-x= partial C4A deficiency

methods section. Table 4.10 gives the chi-square values which indicate the goodness of fit of the model for estimating the probability of an individual having SLE or being healthy, so the higher the chi-square value, the worse the fit. As can be seen in Table 4.10, when C4A alone was included, $X^2_2=4.6$, $p=.098$. When DR3 was included with C4A, the X^2 value was significant, suggesting that DR3 was not contributing to the disease, and when DR3 alone was considered, the chi-square value was highly significant ($p=.001$) showing that the use of DR3 alone did not give a satisfactory model to account for the disease, and could not be the primary factor contributing to SLE when compared with complete C4A deficiency.

The logistic regression results comparing partial C4A deficiency and DR3 are shown in Table 4.11. When C4A was used alone, $X^2_2=3.5$, and $p=.173$, which indicated that the use of partial C4A deficiency alone gave a well fitting model and so accounted for the disease. When both partial C4A deficiency and DR3 were included in the logistic regression, the chi square value for goodness of fit approached significance and when DR3 alone was used, the chi square value was significant ($p=.017$) suggesting that DR3 did not give a satisfactory model and so was not accounting for the disease. Therefore, partial C4A deficiency was more important than DR3 in contributing to the pathogenesis of SLE.

4.3.3 C4B DEFICIENCY VERSUS DR3 IN SLE.

Patients and controls were divided into the four groups shown in Table 4.12, as was done for the C4A analysis. Table 4.13 gives the logistic regression results comparing DR3 and complete C4B deficiency. When C4B was used alone, $X^2_2=14.6$, $p=.001$, showing that complete C4B

Table 4.12 C4B*Q0 AND DR3 IN CAUCASOID SLE PATIENTS AND CONTROLS.

C4B TYPE	SLE		CONTROLS	
	DR3+	DR3-	DR3+	DR3-
Two C4B null alleles	0	2	2	9
One C4B null allele	3	7	26	100
No C4B null alleles	21	13	42	111
Undetermined	12	22	47	170
Total	36	44	117	390

Table 4.13 COMPLETE C4B DEFICIENCY VERSUS DR3.

VARIABLES IN MODEL	X ² (GOF)**	DF*	p
C4B*Q0-Q0 ⁺	14.6	2	0.001
C4B*Q0-Q0, DR3	2.1	1	0.147
DR3	2.1	2	0.347

* DF= degrees of freedom ** GOF= goodness of fit
+ C4B*Q0-Q0= complete C4B deficiency

Table 4.14 PARTIAL C4B DEFICIENCY VERSUS DR3.

VARIABLES IN MODEL	X ² (GOF)**	DF*	p
C4B*Q0-x ⁺	14.4	2	0.001
C4B*Q0-x, DR3	1.4	1	0.237
DR3	7.3	2	0.026

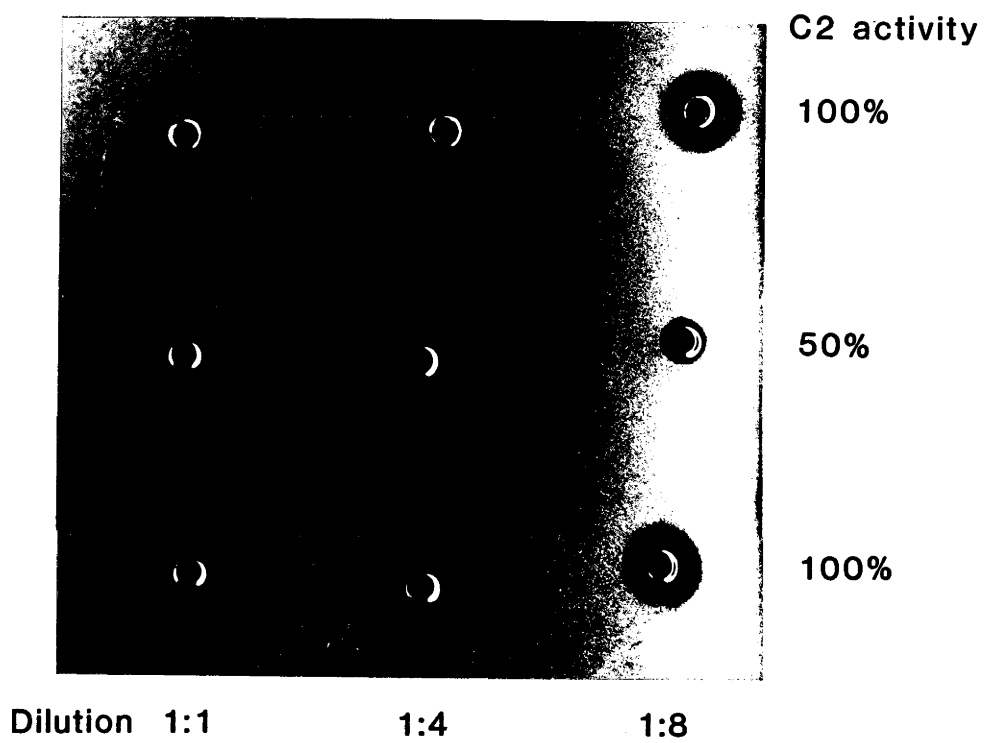
* DF= degrees of freedom ** GOF= goodness of fit
+ C4B*Q0-x= partial C4B deficiency

deficiency did not give a model which accounted for the disease.

However, when DR3 was analysed, the chi-square value for goodness of fit was not significant suggesting that DR3 (or the linked C4A null gene) gave a satisfactory model to account for SLE. When both DR3 and C4B deficiency were included together, there was little change in the chi square value compared with DR3 alone, implying that complete C4B deficiency has little effect on the pathogenesis of the disease.

Logistic regression results comparing the contribution of partial C4B deficiency and DR3 are given in Table 4.14. When C4B alone was included, $\chi^2_2=14.4$, $p=.001$, indicating that use of partial deficiency of C4B alone did not give a good model which accounted for lupus. When DR3 alone was examined, the chi square value was still significant, although not as high as when C4B alone was used, so DR3 (or the linked C4A*Q0 gene) accounted for some, but not all, the disease. When both DR3 and partial C4B deficiency were included, the χ^2 value was not significant, showing that together these variables gave a satisfactory model which accounted for the disease. This can be explained by examining the raw data (Table 4.12). It can be seen in Table 4.12 that 30% of the SLE patients have one C4B null allele and are DR3 positive compared to 20.6% of the controls, which is not significantly different. However, the number of individuals with no C4B null alleles (C4B definite heterozygotes) and with DR3 is much higher in the patient group than the controls, (47.7% as opposed to 27.5%), which is significant ($\chi^2_1=11.3$, $p<.001$). These C4B heterozygotes have either two different C4B alleles or the same two C4B alleles and carry one C4A null allele. So in the logistic regression, when looking at partial C4B deficiency (Table 4.14), DR3 accounts for some of the disease (due to linkage disequilibrium with C4A*Q0), while C4B, (due to many of the C4B

FIGURE 4.2 Photo of a C2 radial diffusion assay showing
100% and 50% C2 activity.



heterozygotes carrying C4A null alleles), appears to account for the rest of the disease.

Therefore, this logistic regression analysis comparing the contribution of C4B deficiency and DR3 to lupus actually appears to support C4A deficiency as playing the major role.

Logistic analysis examining C4A*Q0, C4B*Q0 and DR3 together could not be carried out because of the way in which the null alleles were defined. For example, a person with partial deficiency of C4A must by our criteria of definition have two functional C4B alleles.

4.3.4 C2 DEFICIENCY IN CAUCASOID, JAPANESE AND CHINESE SLE PATIENTS AND CAUCASOID CTD PATIENTS.

Figure 4.3 shows a photo of a C2 haemolytic assay. Assays were performed with undiluted and diluted plasma as indicated and rings of lysis can clearly be seen around the wells. These can be measured by eye or with a ruler to determine the C2 levels in the plasma. Because the normal range of C2 levels is narrow, C2 levels which are below normal can easily be detected (Raum et al. 1980).

Table 4.15 gives C2 levels in the Caucasoid SLE and CTD patients and controls. ^{Low} ^{levels} ^{were} ~~Heterozygous C2 deficiency was~~ seen in 23% of the Caucasoid SLE patients studied here but not in any of the 40 controls ($\chi^2_1=9.1$, $p<.01$) (Table 4.15). A report by Glass et al. (1976) shows that heterozygous C2 deficiency is only seen in 1% of Caucasoids in the general population, so the level seen in the SLE patients studied here is significantly above this. Heterozygous C2 deficiency was also seen in four of fifty (8%) of the Japanese SLE patients and six of fifty (12%) of the Hong Kong Chinese SLE patients. Some of the Caucasoid

Table 4.15 C2 LEVELS IN CAUCASOID SLE AND CONNECTIVE TISSUE DISEASE PATIENTS.

	CONTROLS		SLE		PSS/CREST		SJOGREN'S		PM/DM		MCTD/UNCTD	
C2 level	N	%	N	%	N	%	N	%	N	%	N	%
0%	0		0		0		0		0		0	
50%	0		19	22.9	2	5.9	0		1	5.3	2	12.5
75%	0		2	2.4	2	5.9	0		0		0	
100%	40	100.0	62	74.7	30	88.2	7	100.0	18	94.7	14	87.5
Number Tested	40		83		34		7		19		16	

Table 4.16 C2 PHENOTYPE DISTRIBUTIONS IN CAUCASOID SLE AND CONNECTIVE TISSUE DISEASE PATIENTS.

C2 Phenotype	SLE		PSS/CREST		SJOGREN'		PM/DM		MCTD/UNCTD	
	N	%	N	%	N	%	N	%	N	%
C2-1	39	95.1	14	93.3	3	100.0	5	100.0	7	87.5
C2-1,2	2	4.9	1	6.7	0		0		1	12.5
Number tested	41		15		3		5		8	

Table 4.17 C2 LEVELS IN RELATION TO HLA-B18 AND DR2. IN SLE AND CTD PATIENTS.

HLA HAPLOTYPE																
C2 LEVEL	B18,DRx*				Bx ⁺ ,DR2				B18,DR2				Bx,DRx			
	SLE		CTD		SLE		CTD		SLE		CTD		SLE		CTD	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
50%	0		2	2.5	4	4.8	1	1.3	1	1.2	1	1.3	14	17.1	1	1.3
75%	0		0		0		0		0		0		2	2.4	2	2.5
100%	2	2.4	6	7.5	16	19.5	11	13.8	1	1.2	2	2.5	42	51.2	53	66.3
Total	2		8		20		12		2		3		58		83	

* DRx= not DR2 + Bx= not B18

patients had C2 levels somewhere between 50% and 100% of normal (designated as 75%) which is probably a consequence of the disease.

The CTD patients did not show any significant increases in C2 deficiency although 50% levels were seen in 13% of the MCTD/UNCTD disease group (Table 4.15). However the sample sizes are small (particularly in the Sjogren's and MCTD/UNCTD groups) and a larger patient series will need to be studied to obtain an accurate picture of the role of C2 deficiency in these connective tissue diseases.

Homozygous C2 deficiency was seen in only two patients, both Lebanese, one with SLE and the other with a connective tissue disease, but currently under review. The SLE patient came from a first cousin marriage and was tissue typed as HLA A2,-, B18,-, DR5,-, while the latter patient was not homozygous at the HLA class I loci being typed as HLA A24,-, B18,w49, DR5,-. Both patients also showed very low levels of C4, although the C4 genes were present as indicated by hybridizing DNA from these patients with a C4 cDNA probe, and there is the possibility that the C2 deficiency, could have been caused by some other factor, such as C1 esterase inhibitor deficiency.

The C2 phenotyping results are shown in Table 4.16. No unusual distributions were seen, with C2*1 (formerly known as C2*C) the most common allele in all the patient groups. There were a few individuals with C2*1,2 (C2*C,B) but no rare C2 alleles were seen.

C2 levels were examined in relation to the HLA antigens B18 and DR2 because of the known linkage disequilibrium between C2 deficiency and the haplotype HLA A25.B18.BF*S.C4A*4.C4B*2.DR2(Dw2) (Fu et al. 1975; Hauptmann et al. 1982). However, no association was seen between C2 deficiency and B18 or DR2 in the SLE patients studied here as shown in Table 4.17. However, when C2 levels were examined according to HLA-DR

TABLE 4.18 C2 LEVELS IN RELATION TO HLA-DR TYPE IN SLE AND CTD PATIENTS.

DR TYPE	C2 LEVEL											
	50% C2				75% C2				100% C2			
	SLE		CTD		SLE		CTD		SLE		CTD	
	N	%	N	%	N	%	N	%	N	%	N	%
HLA-DR1	7	36.8	1	20.0	0		1	33.3	7	11.3	15	20.8
HLA-DR2	5	26.3	2	40.0	0		1	33.3	17	27.4	13	18.1
HLA-DR3	3	15.8	0		1	50.0	0		31	50.0	27	37.5
HLA-DR4	5	26.3	3	60.0	0		2	66.7	15	24.2	19	26.4
HLA-DR5	4	21.1	2	40.0	1	50.0	0		11	17.7	19	26.4
HLA-DR7	5	26.3	0		1	50.0	1	33.3	14	22.5	11	15.3
HLA-DRw8	0		0		0		0		1	1.6	6	8.3
HLA-DRw9	0		0		0		0		0		3	4.2
HLA-DRw10	0		0		0		0		0		1	1.4
HLA-DRw13	3	15.8	0		1	50.0	0		10	16.6	6	8.3
HLA-DRw14	2	10.5	0		0		0		2	3.2	5	6.9
Total	19		5		2		3		62		72	

type, HLA-DR1 appeared to be associated with partial C2 deficiency in the SLE patients, but this was not statistically significant when corrected for the number of antigens tested for. (Table 4.18). A negative association was seen with HLA-DR3 and partial C2 deficiency. The CTD patients did not show any association between partial C2 deficiency and HLA-DR.

C2 levels were crosstabulated with the number of C4A or C4B alleles present to see if there was any association between low C2 and low C4 levels. Uko et al. (1986) have reported that the serum concentration of C4 can be correlated with the number of C4 alleles, so C2 levels were examined according to whether individuals were C4A or C4B null homozygotes, C4A or C4B heterozygotes, C4A or C4B null heterozygotes, or, C4A or C4B undetermined heterozygotes or homozygotes. C4A and C4B null heterozygotes were assigned according to density differences between the C4A and C4B alleles. No association was seen between C2 levels and the number of C4 alleles indicating that low C2 levels probably do not correlate with low C4 levels. Three of the 19 SLE patients with 50% C2 levels also had very low C4 levels that made C4 allotyping impossible, but whether these low C2 and low C4 levels are associated could not be determined.

4.4 DISCUSSION.

4.4.1 SIGNIFICANCE OF C4A*Q0 IN SLE PATIENTS.

This study of C4 distributions in three ethnic groups has shown a systematic increase in the frequency of C4A null alleles in SLE patients. This increased C4A null gene frequency is due not only to an

excess of C4A null homozygotes, but also to an increase in C4A null carriers in the patient groups. C4A null homozygosity was significantly increased in Caucasoid and Japanese patients while the number of C4A null carriers or apparent homozygotes was increased in Caucasoid and Chinese patients. Thus complete and partial deficiencies of C4A are implicated in SLE, significantly increasing the C4A*Q0 gene frequency in SLE patients from three ethnic groups.

Although partial complement deficiency has been implicated previously in the predisposition to SLE in Caucasoids (Fielder et al. 1983) the relative importance of C4A and C4B null alleles as disease determinants remains controversial (Christiansen et al. 1983). In addition, because the C4 alleles are in strong linkage disequilibrium with the HLA alleles, with C4A*Q0 carried on the HLA-A1.B8.DR3 haplotype and C4B*Q0 on the HLA-B18.DR3 haplotype (Christiansen et al. 1983; Revielle et al. 1985) it has been difficult to distinguish between the HLA class II and class III genes as the primary factors involved in predisposition to SLE.

HLA-DR associations with SLE do tend to differ between and within different ethnic groups, suggesting that HLA-DR antigens may not be the causative factors in SLE. For instance, in Caucasoid SLE patients, increased frequencies of both HLA-DR3 (Celada et al. 1979; Dunckley et al. 1986) and HLA-DR2 have been reported (Reinertsen et al. 1978; Gladman et al. 1979; Reveille et al. 1985), while in Japanese HLA-DR2 was increased in one study, (Hashimoto et al. 1985), but not in another where the only significant change in DR distributions was a decrease in DR4 (Kameda et al. 1982). A recent study of SLE in Hong Kong Chinese has shown an increase in HLA-DR2 (Hawkins et al. 1987). These different HLA-DR associations suggest MHC-linked determinants rather than HLA-DR

antigens, are involved in the pathogenesis of SLE. In addition to the ethnic study, logistic regression analysis here has clearly shown that both complete and partial C4A deficiency are more important than DR3 in contributing to SLE. C4B deficiency (either complete or partial) is not implicated in the pathogenesis of SLE in this study. This is supported by a recent study looking at C4 in white and black Americans which also showed an increase in C4A*Q0 but not C4B*Q0 in SLE patients in both ethnic groups ($p < .05$) (Howard et al. 1986). In addition, Green et al. (1986) using the empirical logistic method, presented results indicating that the association between C4A*Q0 and SLE is primary while that of DR3 and SLE is secondary. A recent study by Kemp et al. (1987) used Southern blotting to look at the C4 genes in SLE and found a high proportion of patients with deletions in the C4A gene.

The reason for the association of SLE with C4A*Q0 and not C4B*Q0 may reflect the different functional properties of C4A and C4B. C4 is activated through cleavage of the internal thiolester bond, and it is able to acylate hydroxyl, amino or other nucleophilic groups of proteins, carbohydrates or other antigenic molecules. C4A has been shown to bind more readily to amino groups in contrast to C4B which binds more readily to hydroxyl groups (Isenman and Young 1984; Law et al. 1984). Thus C4A may be more efficient than C4B in denaturing the protein immune complexes which characterize SLE. A study by Schifferli et al. (1986b) supports this by showing that C4A is 1.7 fold more efficient than C4B in preventing immune precipitation, and therefore plays an important role in prevention of immune complex precipitation and the clearance of immune complexes. Therefore, a decrease in serum C4B would have less impact than a decrease in serum C4A. Partial C4A deficiency could be accentuated because C4A competes with C4B as a

substrate for C1, and C4B would be in 2 fold excess over C4A, therefore even less C4A would be activated (Atkinson 1986). In addition, Sim and Law, (1985), in the study of hydralazine induced SLE, have shown that hydralazine binds to C4A more efficiently than C4B thereby inactivating it which is consistent with the proposal that the lack of C4A is more important than C4B in determining susceptibility to SLE.

If this proposition is correct, complete and partial C4A deficiencies should be genetic determinants of SLE not only in Caucasoids but also in other ethnic groups. This study has confirmed an increase in frequency of C4A null alleles in Caucasoid SLE patients and has shown a similar increase in Chinese and Japanese SLE patients also. Since only one third of the SLE patients have complete or partial deficiency of C4A, this cannot be the only determinant of SLE.

No increase of the C4A*6 allele was seen in the Caucasoid, Japanese or Chinese SLE patients. C4A*6 can have less in vitro haemolytic activity than the other C4 alleles because a less active C5 convertase is formed (Dodds et al. 1985). However, if the reaction of the C4A protein with amino nucleophiles is the initial, important step in clearing protein immune complexes, a lower level of C5 convertase activity may not seriously affect in vivo function.

4.4.2 C2 IN SLE.

There was an increased frequency of partially C2 deficient individuals in the SLE patients of Caucasoid, Japanese and Chinese origin which is in accord with other studies which have associated C2 deficiency with SLE (Glass et al. 1976; Schur 1982). The classical complement pathway is essential for the prevention of precipitation of

immune complexes (Webb and Whaley 1986) so lack of C2 would probably result in less efficient processing of immune complexes, more immune complexes precipitating and therefore becoming harder to solubilize, and increased deposition of immune complexes in the tissues where they could cause damage. Further local tissue damage would also result if the complement system was activated by the deposited immune complexes.

The lack of association between C2 deficiency and HLA-B18 or DR2 seen here is in contrast to other studies which have reported a high degree of linkage between the extended haplotype HLA A25.B18.BF*S.C4A*4.C4B*2.DR2(Dw2) and C2 deficiency (Hauptmann et al. 1982; Schur 1982). A report by Christiansen et al. (1982) indicated that their lupus patients, like those here, did not show an association between C2 deficiency and the extended haplotype above. Six of the 39 SLE patients were HLA B18 positive, but not C4A*4.C4B*2.BF*S positive suggesting they were not C2 deficient. Three of the 13 B18 positive controls (from a total of 176 controls) were C4A*4.C4B*2.BF*S positive. It is possible that some of the C2 deficiencies seen here are acquired as a result of the disease and are not due to a defective or null gene, and therefore would not be expected to show linkage to genes in the MHC. While nearly 40% of individuals with homozygous C2 deficiency develop SLE (Agnello et al. 1986), partial inherited deficiency of C2 may not be so detrimental.

4.4.3 C4 AND C2 IN CONNECTIVE TISSUE DISEASES.

In contrast to the SLE patients, none of the CTD patient groups showed an increase in C4A*Q0 frequency apart from the MCTD/UNCTD patients but only 4 MCTD and 15 UNCTD patients were studied. A study of

patients with scleroderma (Briggs et al. (1986), has shown a significant increase in C4A*Q0 in the patients, however no association was seen here. The Pm/Dm patients showed an increased frequency of C4A*4 and C4B*2 which are known to be in linkage disequilibrium on the extended haplotype HLA-A25.B18.BF*S.C2*Q0.DR2(Dw2) (Hauptmann et al. 1982). However, the Pm/Dm patients did not show an increase in C2 deficiencies (Table 4.9) indicating that the C4A*4 and C4B*2 alleles may not be carried on the same chromosome. Alternatively, it is possible that the C4A*4 and C4B*2 alleles are markers for some other gene which is in linkage disequilibrium with them. The C4B null gene did not appear to be a risk factor for the CTDs studied here. Mollenhauer et al. (1984) in a report of patients with scleroderma showed an increased frequency of C4B*Q0 homozygotes in their patient group, but this was not seen in the scleroderma patients here.

A role for C2 deficiency was not supported in the connective tissue diseases studied here as the CTD patients did not show any significant increases in C2 deficiency unlike the SLE patients.

4.5 CONCLUSION.

SLE is a clinically heterogeneous autoimmune disorder and this study suggests that there is genetic heterogeneity in its aetiology. However, one important genetic determinant of SLE in Caucasoid, Japanese and Chinese populations is complete or partial deficiency of complement component C4A. In addition, logistic regression in Caucasoids has shown that both complete and partial C4A deficiency is more important than DR3 in contributing to lupus. Partial C2 deficiency was increased in the SLE patients studied here, but this may be a consequence of the disease

and not an inherited genetic defect. While exacerbating the disease, partial C2 deficiency may not be as serious as C4A deficiency.

From the small numbers in this study, it would appear that deficiencies of complement components C2 and C4 do not play a major role in the pathogenesis of PSS, CREST, Pm/Dm, MCTD, UNCTD, or Sjogren's syndrome.

CHAPTER FIVE

THE T-CELL RECEPTOR IN SLE

5.1 INTRODUCTION.

The T-cell antigen receptor is responsible for recognizing foreign antigens either in association with the MHC class I antigens, (the $\alpha\beta$ receptor in association with CD8 on T-cytotoxic cells), or class II HLA-D antigens, (the $\alpha\beta$ receptor found on T-helper cells in association with CD4). The foreign antigen is firstly processed by cells such as B-cells or antigen presenting cells, (Grey and Chestnut 1985; Germain 1986; Mills 1986), and is then presented on the cell surface in association with the HLA class I or II proteins (Babbitt et al. 1986; Phillips et al. 1986), where it is recognized by the T-cell receptor. The T-cell is then activated and can further stimulate the immune response (T-helper cell) by stimulating B-cell activation resulting in antibody class switching (IgM to IgG) and immune "memory". Alternatively, cytotoxic T-cells or killer T-cells can be activated to kill cells carrying the foreign antigen. If the T-cell receptor was defective and was reactive with self antigens or was not MHC restricted, an immune response to self antigens may be initiated which could result in autoantibodies. Another possibility is that the TCR may fail to recognize foreign antigen such as a virus which could lead to a persistent infection.

5.1.1 THE T-CELL RECEPTOR IN AUTOIMMUNITY.

A possible role for the TCR genes in SLE is suggested by studies of NZW mice and their offspring. In 1985, Kotzin et al. showed with RFLP analysis that the NZW mouse had a deletion of 8.8 kb in the TCR- β gene encompassing the C β 1, J β 2 and D β 2 loci. This has been shown to start in the first 167 bp of the first C gene and extend to the homologous area

of the second C gene (Noonan et al. 1986). This reduces the potential diversity of the T-cell receptor and even though all the $\alpha\beta$ TCR's in NZW mice must be derived from a single set of TCR- β genes they are phenotypically normal. A subsequent paper, (Yanagi et al. 1986), analysed the contribution of the TCR- β deletion to the autoimmunity seen in (NZW x NZB)F1 offspring, which develop a lupus like disease. The (NZW x NZB)F1 hybrids were crossed with NZB mice and the offspring showed a high association between autoantibody formation and circulating retroviral gp70 immune complexes and the TCR- β deletion or a locus closely linked to it. The appearance of SLE in the F1 offspring suggests a combinatorial or interactive role for at least two loci, one of which may encode the TCR- β chain and the other, as shown in (NZB x F1) backcrosses, an MHC-linked gene (Kotzin and Palmer 1987).

5.1.2 AIMS.

This chapter examines RFLPs associated with the T-cell receptor α , β , and γ chain genes in SLE and associated connective tissue diseases, to search for unusual gene rearrangements. In addition, possible interaction of the TCR RFLPs with HLA-DR and -DQ RFLPs in SLE is examined.

5.2 METHODS.

5.2.1 PATIENTS AND CONTROLS.

Caucasoid patients with SLE and the connective tissue diseases Sjogren's syndrome, scleroderma, linear scleroderma, CREST, Pm/Dm, mixed

connective tissue disease and undifferentiated connective tissue disease were studied. Controls comprised healthy blood bank donors and laboratory personnel.

5.2.2 PROBES.

The TCR- α cDNA clone pY14 (1100bp) (Yanagi et al. 1985) which encodes a complete TCR- α protein was used. The TCR- β cDNA clone (770 bp) contains the J and C region (Yoshikai et al. 1984), and the TCR- γ cDNA clone contains the V, J, and C- γ 1 region (Dr. T. Mak, personal communication). The three probes were provided by Dr. T. Mak.

5.2.3 RESTRICTION ENZYMES.

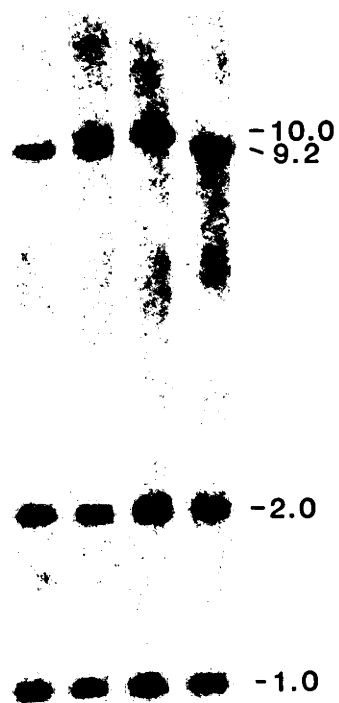
The following TCR probe and restriction enzyme combinations were examined in patients and controls to find useful RFLPs for this study.

A. TCR- α . BglII digested DNA revealed no RFLPs in 80 patients while EcoRI did show two polymorphic fragments in 20 patients of approximate size 17kb and 9kb, but EcoRI was not a reliable enzyme and therefore was not used. EcoRV digested DNA showed two useful RFLPs and DNA was readily digested to completion with this enzyme. So et al. (1987) reported that TaqI digested DNA was polymorphic with the TCR- α probe and this was also seen in our patients. EcoRV and TaqI appeared to be the most useful enzymes with the TCR- α probe and were used in this study.

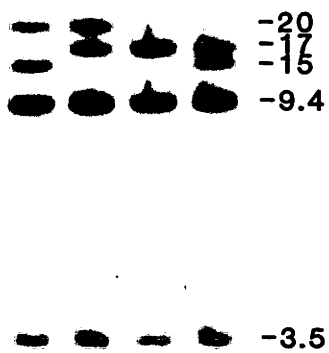
B. TCR- β . It has been observed that DNA digested with BglII is polymorphic with TCR- β (Robinson and Kindt 1985; Berliner et al. 1985) and this restriction enzyme was used here. EcoRI digested DNA showed a RFLP in only one patient out of twenty and therefore was not used.

FIGURE 5.1 a. TCR β hybridization of BglII digested DNA.
b. TCR γ hybridization of PvuII digested DNA.
c. TCR γ hybridization of TaqI digested DNA.

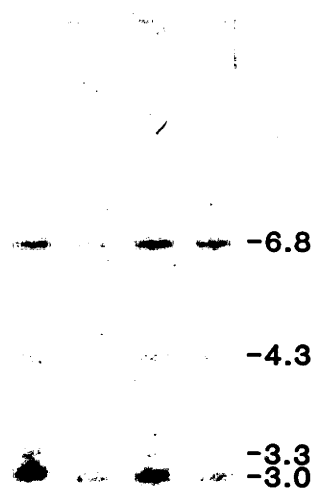
a. TCR β /BglIII



b. TCR γ /PvuII



c. TCR γ /TaqI



C. TCR- γ . While EcoRV digested DNA did show two polymorphic fragments approximately 23 and 24 kb in size, this enzyme was not used because of the difficulty in distinguishing between the two fragments. DNA digested with BglIII showed no RFLPs in 20 patients or 7 controls. The following enzymes showed no polymorphisms in a small number of controls: AvaII (5 controls), HindIII (4), MspI (4), and PstI (6), but PvuII digested DNA revealed three RFLPs in 7 controls and was used in this study. TCR- γ was also polymorphic with TaqI as seen initially in 40 patients and was used in this study in addition to PvuII.

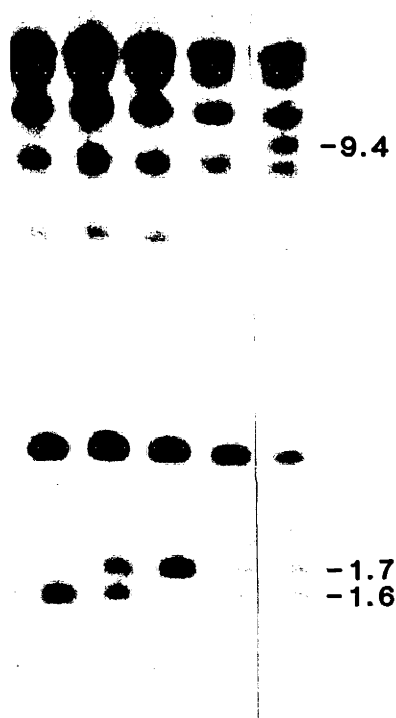
5.3 RESULTS.

5.3.1 TCR- α , - β , and - γ RFLPS.

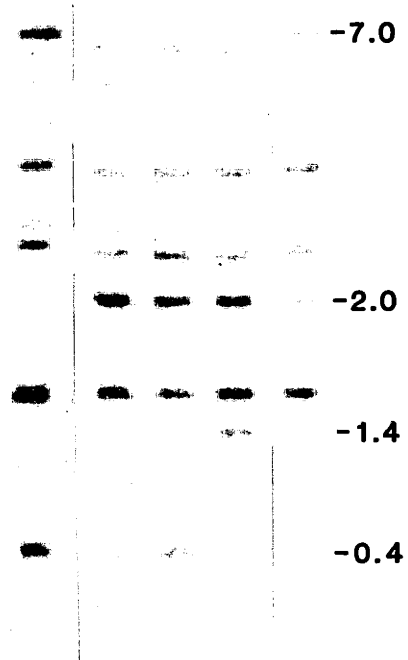
Figure 5.1 shows polymorphic fragments observed with TCR- β and - γ cDNA probes. The TCR- β /BglIII polymorphism of allelic 10.0kb and 9.2kb fragments has been described previously (Robinson and Kindt 1985). Two constant fragments were also evident, approximately 2.0kb and 1.0kb in size. For TCR- γ , as reported by Dunckley et al. (1988), PvuII generated an informative polymorphism with three allelic fragments 20kb, 17kb and 15kb in size and two constant fragments of 9.4kb and 3.5kb. TCR- γ was also polymorphic when hybridized with TaqI digests of genomic DNA, with a 3.3kb fragment either present or absent. Seven non-polymorphic fragments were also present in the TCR- γ /TaqI hybridization, with fragments about 6.8kb, 4.3kb and 3.0kb hybridizing strongly and fragments approximately 6.2kb, 2.2kb, 1.3kb, and 0.8kb hybridizing less strongly. Figure 5.2 shows the RFLPs observed with the TCR- α probe. EcoRV digests were informative with allelic fragments of 1.7kb and

FIGURE 5.2 a. TCR α hybridization of EcoRV digested DNA.
b. TCR α hybridization of TaqI digested DNA.

a. TCR α /EcoRV



b. TCR α /TaqI



1.6kb, as well as an unlinked 9.4kb polymorphic fragment (Dunckley et al. 1988). In addition, constant fragments approximately 23kb, 19kb, 14kb, 8.5kb, 5.5kb and 2.5kb in size were observed. TCR- α is also polymorphic with TaqI (So et al. 1987) with allelic fragments of 7.0kb and 2.0kb due to changes in restriction sites in the constant TCR- α gene and a 1.4kb fragment either present or absent due to a TCR- α variable gene restriction site polymorphism. In this study, an additional polymorphic fragment was seen, approximately 0.4kb in size. Eight non-polymorphic fragments are also evident in the TCR- α /TaqI hybridization.

5.3.2 TCR- β IN THE SLE AND CTD PATIENTS.

Table 5.1 shows the TCR- β /BglII phenotype and allele frequencies in the patient groups and controls. The SLE patients showed no significant differences from the controls in their phenotype distributions ($\chi^2_2=0.54$, $p=.8$) or allelic frequencies ($\chi^2_1=0.54$) of the 10.0kb and 9.2kb fragments. The CTD patients did show a difference in phenotype and allele distributions, with all patient groups showing a trend towards an increase of the 10.0kb fragment with a significant increase in the MCTD/UNCTD disease group ($p(\text{corr})=.05$). However, the patient numbers in the individual CTD groups are small and a larger patient series would be needed to verify this increase. Interestingly, when all the CTD patients were combined, the increase in the 10.0kb fragment was significant ($\chi^2_1=6.5$, $p=.01$).

TABLE 5.1 TCR- β /BglII PHENOTYPES IN SLE AND CTD PATIENTS COMPARED WITH CONTROLS.

Phenotype	Controls		SLE		PSS/CREST		Pm/Dm		MCTD/UNCTD		Sjogren's	
	N	%	N	%	N	%	N	%	N	%	N	%
10.0/10.0	9	21.4	18	27.7	5	31.3	3	37.5	4	30.8	2	33.3
10.0/9.2	20	47.6	29	44.6	9	56.3	4	50.0	9	69.2	4	66.7
9.2/9.2	13	31.0	18	27.7	2	12.5	1	12.5	0		0	
Allele frequency												
10.0 kb	0.452		0.500		0.594		0.625		0.653		0.667	
9.2 kb	0.548		0.500		0.406		0.375		0.346		0.333	
Number tested	42		65		16		8		13		6	

TABLE 5.2 TCR- γ /PvuII PHENOTYPES IN SLE AND CTD PATIENTS COMPARED WITH CONTROLS.

Phenotype	Controls			SLE			PSS/CREST			Pm/Dm			MCTD/UNCTD			Sjogren's		
	N	%		N	%		N	%		N	%		N	%		N	%	
20/20	1	2.0	3	3.8	2	5.7	2	5.7	2	14.3	0					0		
20/17	13	26.0	18	22.5	6	17.1	2	14.3	5	25.0	0					0		
20/15	5	10.0	9	11.3	2	5.7	1	7.1	2	10.0	0					0		
17/17	21	42.0	24	30.0	18	51.4	4	28.6	7	35.0	4	66.7						
17/15	8	16.0	21	26.3	5	14.3	3	21.4	5	25.0	2	33.3						
15/15	2	4.0	5	6.3	2	5.7	2	14.3	1	5.0	0							
Allele frequency																		
20 kb	0.200		0.206		0.171		0.250		0.175		0.000							
17 kb	0.630		0.544		0.671		0.464		0.600		0.833							
15 kb	0.170		0.250		0.157		0.286		0.225		0.167							
Number tested.	50		80		35		14		20		6							

5.3.3 TCR- γ IN THE SLE AND CTD PATIENTS.

Table 5.2 gives phenotype distributions and allele frequencies for the TCR- γ /PvuII RFLPs. There were no differences seen in the allele ($\chi^2_2=1.9$) and phenotype ($\chi^2_5=3.53$, $p=.06$) distributions between the SLE patients and the controls. Similarly, the CTD groups did not show any significant differences from the controls in allele and phenotype distributions either in the individual disease groups or when combined, with all phenotype distributions in Hardy-Weinberg equilibrium. Distribution of the 3.3kb TCR- γ /Taq I RFLP in patients and controls is shown in Table 5.3. The 3.3kb fragment was present at a similar frequency in both the controls and SLE patients. Of the connective tissue diseases, only the MCTD/UNCTD group showed a significant increase in the presence of this fragment ($p<.05$). This was due to an increase in the UNCTD disease group with 7/13 patients (53.8%) ($p<.05$) having this fragment. No linkage disequilibrium was seen between the TCR- γ /TaqI 3.3kb fragment and the TCR γ /PvuII 20kb fragment ($\chi^2_1=1.3$, $p(\text{corr})=0.2$), or the TCR γ /PvuII 17kb fragment ($\chi^2_1=0.0$, $p(\text{corr})=1.0$), or the TCR γ /PvuII 15kb fragment ($\chi^2_1=2.9$, $p(\text{corr})=.09$).

5.3.4 TCR- α IN THE SLE AND CTD PATIENTS.

Phenotype and allele frequencies of the TCR- α /EcoRV RFLPs in patients and controls are given in Table 5.4. The SLE patients showed no significant differences when compared with the controls in their phenotype ($\chi^2_2=0.29$, $p=0.9$) or allele frequencies ($\chi^2_1=0.23$). The CTD groups also showed similar phenotype and allelic distributions as the controls and no significant differences were seen even when the CTD

TABLE 5.3 FREQUENCY OF THE TCR- γ /TaqI 3.3kb FRAGMENT IN SLE AND CTD PATIENTS AND CONTROLS.

Phenotype	Controls		SLE		PSS/CREST		Pm/Dm		MCTD/UNCTD	
	N	%	N	%	N	%	N	%	N	%
TCR- γ /TaqI 3.3 kb	9	15.3	16	23.9	8	30.8	2	15.4	8*	47.1
Number tested	59		67		26		13		17	

*p<.05

TABLE 5.4 TCR- α /EcoRV PHENOTYPES IN SLE AND CTD PATIENTS COMPARED WITH CONTROLS.

Phenotype	Controls		SLE		PSS/CREST		Pm/Dm		MCTD/UNCTD		Sjogren's	
	N	%	N	%	N	%	N	%	N	%	N	%
1.7/1.7	2	4.8	4	7.1	2	10.0	0		1	6.3	0	
1.7/1.6	16	38.1	22	39.3	6	30.0	6	60.0	5	31.3	3	60.0
1.6/1.6	24	57.1	30	53.6	12	60.0	4	40.0	10	62.5	2	40.0
Unlinked 9.4kb RFLP	6	14.3	8	14.3	1	5.0	2	20.0	1	6.3	1	20.0
Allele frequency												
1.7 kb		0.238		0.263		0.250		0.300		0.219		0.300
1.6 kb		0.762		0.732		0.750		0.700		0.781		0.700
Number tested	42		56		20		10		16		5	

TABLE 5.5 TCR- α /TaqI PHENOTYPES IN SLE AND CTD PATIENTS
COMPARED WITH CONTROLS.

	Controls		SLE		PSS/CREST		Pm/Dm		MCTD/UNCTD	
Phenotype	N	%	N	%	N	%	N	%	N	%
7.0/7.0	4	10.8	3	10.0	1	14.3	1	20.0	1	25.0
7.0/2.0	14	37.8	15	50.0	6	85.7	2	40.0	3	75.0
2.0/2.0	19	51.4	12	40.0	0		2	40.0	0	
Allele frequency										
7.0 kb		0.297		0.350		0.571		0.400		0.625
2.0 kb		0.703		0.650		0.429		0.600		0.375
Number tested		37		30		7		5		4

TABLE 5.6 FREQUENCY OF THE TCR- α /TaqI 1.4kb FRAGMENT IN SLE AND CTD
PATIENTS COMPARED WITH CONTROLS.

	Controls		SLE		PSS/CREST		Pm/Dm		MCTD/UNCTD	
Phenotype	N	%	N	%	N	%	N	%	N	%
TCR-TaqI 1.4 kb	19	54.3	9	42.9	2	22.2	4	66.6	2	100.0
Number tested		35		21		9		6		2

patients were combined. Table 5.5 shows the allele and phenotype frequencies of the TCR- α /TaqI RFLPs in the SLE and CTD patients and controls. Again, no significant differences were seen in the phenotype ($\chi^2_2=1.03$, $p=0.6$) and allele ($\chi^2_1=0.37$) distributions between the SLE patients and controls. Likewise, the phenotype and allelic distributions of the TCR- α /TaqI RFLPs were similar between the CTD patients (in the individual disease groups and when combined) and the controls. Distribution of the TCR- α /TaqI 1.4kb fragment is shown in Table 5.6. The 1.4kb fragment was present at a similar level in the SLE patients and controls. The patient numbers in the CTD groups studied here were low, but no significant differences were seen between the CTD groups and the controls in the distribution of the 1.4kb fragment. There was no linkage disequilibrium between the TCR- α /EcoRV 1.7kb fragment and the TCR- α /TaqI 7.0kb ($\chi^2_1=0.18$, $p(\text{corr})=0.6$) or 2.0kb ($\chi^2_1=3.2$, $p(\text{corr})=0.7$) fragments. Chi-square values for linkage disequilibrium between the TCR- α /EcoRV 1.6kb fragment and the TCR- α /TaqI fragments could not be calculated because all individuals included in the linkage disequilibrium analysis were positive for the EcoRV 1.6kb fragment. (Some patients were negative for the EcoRV 1.6kb fragment, but were not included in the analysis because TaqI results were not available).

5.3.5 TCR RFLPS AND HLA-DR PHENOTYPES.

TCR phenotypes were examined according to DR phenotypes of the SLE patients as shown in Table 5.7. The phenotypes DR2,x, DR2,3, and DR3,y were tested because of the reported associations between SLE and DR2 and DR3. DR phenotypes were assigned by DNA typing as described in

TABLE 5.7 TCR PHENOTYPES AND HLA-DR IN SLE.

TCR phenotype	DR phenotype							
	2,X*		2,3		3,Y ⁺		X,Y	
	N	%	N	%	N	%	N	%
TCR- α /EcoRV (n=46)								
1.7/1.7	1	14.3	1	14.3	1	5.9	0	0.0
1.7/1.6	3	42.9	1	14.3	6	35.3	6	40.0
1.6/1.6	3	42.9	5	71.4	10	58.8	9	60.0
Total	7		7		17		15	
TCR- β /BglIII (n=56)								
10.0/10.0	3	50.0	1	20.0	5	25.0	8	32.0
10.0/9.2	2	33.3	3	60.0	8	40.0	13	52.0
9.2/9.2	1	16.7	1	20.0	7	35.0	4	16.0
Total	6		5		20		25	
TCR- γ /PvuII (n=79)								
20/20	1	7.7	0	0.0	1	4.0	1	3.1
20/17	3	23.1	1	11.1	8	32.0	6	18.8
20/15	1	7.7	2	22.2	0	0.0	6	18.8
17/17	2	15.4	2	22.2	8	32.0	11	34.4
17/15	6	46.2	3	33.3	6	24.0	6	18.8
15/15	0	0.0	1	11.1	2	8.0	2	6.2
Total	13		9		25		32	

X: not DR2 Y: not DR3

Chapter 3. No significant differences were seen in T-cell receptor phenotype distributions or allele frequencies between the different DR phenotypes examined, but patient numbers with some of the selected DR phenotypes were small, in particular DR2,x and DR2,3, (allele frequencies not shown).

Table 5.8 shows the allelic frequencies of TCR RFLPs in relation to the HLA-DR and HLA-DQ associated RFLPs of the SLE patients. No significant associations were seen between any of the TCR RFLPs and the HLA-DR or -DQ RFLPs. Possible DR,TCR and DQ,TCR interactions were examined according to presence or absence of a given DR or DQ type (described in Chapter 3) and presence or absence of a given TCR RFLP. The 1.6kb TCR- α /EcoRV fragment and HLA-DRw6 were positively associated ($\chi^2_1=4.31$, $p<.05$), but when corrected for the number of statistical tests made, this was no longer statistically significant.

5.4 DISCUSSION.

5.4.1 THE T-CELL RECEPTOR IN SLE.

This study has shown that there are no major deletions or rearrangements in the T-cell receptor α , β or γ chain genes in SLE patients. Major rearrangements or unusual clonality in TCR genes should be detected with almost any common-cutter restriction enzyme, but no novel RFLPs were seen in patients that were not seen in controls. Similarly, any major deletions in the TCR genes would be evident in altered restriction fragment sizes, or, if the gene was deleted in its entirety, by increased apparent homozygosity for RFLP patterns in the patient series. Minor deletions or alterations in the TCR genes are

less easily detected in Southern blot analysis, but linkage disequilibrium between polymorphic restriction enzyme sites and possible defects in TCR genes would be expected. Neither TCR- α , - β nor - γ RFLPs were associated with SLE in this study. This is in accord with two earlier studies, both with 14 SLE patients, (Fronek et al. 1986, Goldstein et al. 1987) which showed that that TCR- β RFLPs in patients were similar in distribution to those seen in controls. In contrast, a study of rheumatoid arthritis patients showed clonal TCR- β rearrangements in synovial fluid T-cells that were readily detected with a TCR- β constant region cDNA probe (Savill et al. 1987).

Evidence for TCR- β gene involvement in murine SLE models is conflicting. A study of the MRL-lpr/lpr mouse strain which develops an SLE-like autoimmune disease has shown TCR V- β gene expression to be significantly skewed in favour of certain V- β genes suggesting a possible role for the TCR variable genes in autoimmunity and SLE (Singer et al. 1986). A recent study of SWR x NZB mice has shown I-A β and TCR- β or closely linked genes are associated with the development of lupus nephritis in the F1 offspring (Ghatak et al. 1987). While Yanagi et al. (1986) found an association between the TCR- β deletion and SLE in the (NZW x NZB)F1 mouse, Kotzin and Palmer (1987) in their NZB x (NZW x NZB)F1 backcross studies concluded that lupus-like disease is largely associated, although not absolutely, with heterozygosity at an MHC-linked locus, rather than with the TCR- β deletion. NZW mice have been shown to have multiple RFLPs in their TCR- α variable region genes compared with NZB and non-autoimmune mouse strains, (Kotzin and Palmer, 1987) as well as an unusual MHC haplotype, H-2^Z (Klein 1975) so it is possible that there are other loci, as yet undetected, which show

unusual rearrangements in the NZW mice and that these are contributing to the lupus-like disease seen in the NZW x NZB offspring.

The observations reported here discount an important role for inherited differences in TCR- γ , TCR- α and TCR- β genes in contributing to SLE in man since phenotype and allelic distributions are normal and there is no significant increase in any HLA-DR,TCR genic combination, nor any T-cell receptor phenotype distribution differences in the HLA-DR phenotypes DR2,x, DR2,3, or DR3,y.

5.4.2 THE T-CELL RECEPTOR IN CONNECTIVE TISSUE DISEASES.

Similarly, the TCR- γ and TCR- α genes do not appear to play a role in the connective tissue diseases studied here with only one significant difference seen, an increase in the TCR- γ /TaqI 3.3 kb fragment in the UNCTD patients, but the number of patients was small (13) so this association may not be seen in a larger patient series. The CTD patients did show an increase in the 10.0kb TCR- β /BglIII fragment which was significant when all the connective tissue disease patients were grouped together. A larger patient series would be required to examine the possible involvement of the TCR- β gene in predisposing to these connective tissue diseases.

5.4.3 FUTURE T-CELL RECEPTOR RESEARCH.

A possible role for the TCR- γ gene in autoimmunity is suggested by a study which showed a higher level of γ gene expression in autoreactive T-helper cells compared with antigen specific T-helper cells which show a low level of expression (Zauderer et al. 1986). Future study of the

possible role of TCR genes in the pathogenesis of SLE or associated connective tissue diseases may need to examine T-lymphocyte subsets for novel TCR gene arrangements and unusual clonality. The study by Savill et al. (1987) which showed clonal TCR- β gene rearrangements in rheumatoid arthritis examined cells from a specific tissue, the synovium, which would presumably contain T-cell subsets of particular antigenic reactivity. This shows the value of looking at particular subsets of cells. Studies with TCR variable region probes may also prove useful in the study of SLE but the study on rheumatoid arthritis by Savill was done with a probe from the constant region of the TCR- β gene which shows that this should be sufficient to detect unusual rearrangements or clonality in SLE also.

CHAPTER SIX

DISCRIMINANT ANALYSIS IN SLE
AND OTHER IMMUNOLOGICAL VARIABLES.

6.1 INTRODUCTION.

SLE is an extremely heterogeneous disease which can present clinically in many different ways and in differing degrees of severity. Diagnosis of SLE depends on the presence of a minimum of four, out of a total of 20, clinical and laboratory criteria, as established by the American Rheumatism Association (Tan et al. 1982), so it is possible for two given patients to be diagnosed with lupus and yet have no overlapping clinical or laboratory features. Attempts at clinical subsetting of SLE patients, aimed at improved patient treatment and management, have been made (Urowitz 1977; Provost 1979). However, the reproducibility of the proposed clinical and serological subsets is poor (American Rheumatism Association 1983), with patients sometimes shifting from one subset to another during the disease process. A useful approach to defining disease subsets in lupus is that of attempting to define genetic subsets. This approach was paramount in defining subsets of diabetes, with Type 1 (juvenile onset diabetes) clearly MHC associated and Type 2 (adult onset diabetes) not MHC associated (Cudworth et al. 1975).

A further difficulty in defining distinct clinical entities is that SLE patients share some features, including the production of a large array of autoantibodies, with other connective tissue diseases such as CREST, scleroderma, Sjogren's syndrome, dermatomyositis and polymyositis. Once again, the genetic approach may be useful, for example Whittingham et al. (1983a) found a strong association between anti-SS-A (La) and HLA-B8 in Sjogren's syndrome.

One method of looking for genetic associations in SLE is to use discriminant analysis on a large patient group which can be subdivided,

for example by the classification criteria, to see what factors may be important in the susceptibility to certain clinical features. With discriminant analysis, it is also possible to analyse a large number of variables and assess their relative importance to the disease. Further, it is possible to search for the genetic and clinical features that help discriminate SLE from other connective tissue diseases.

Although this study has focussed on the role of the MHC and the T-cell receptor genes in the pathogenesis of lupus, other candidate genes include the immunoglobulins (Whittingham et al. 1983b) and complement component C3 (McLean et al. 1980). Additional features that may or may not be under genetic control, include the presence or absence of lymphocytotoxic antibodies (LCAs) and rheumatoid factor (RF). This chapter describes the distribution of Gm allotypes, C3 phenotypes, LCAs and RF in SLE and CTD patients. These factors are then included along with other genetic and clinical features in discriminant analysis of SLE and other CTDs.

6.1.1 THE IMMUNOGLOBULINS.

Immunoglobulins (Igs) or antibodies are secreted from B-cells in response to foreign antigen and can be one of five classes: IgM, IgG, IgA, IgE, or IgD. They are comprised of four polypeptide chains, two heavy and two light. There are two classes of light genes, kappa (κ) and lambda (λ). Polymorphisms of the immunoglobulins can be detected serologically, with those of the IgG heavy chain known as Gm allotypes, and those of κ called Km. The Gm allotypes are commonly inherited as haplotypes which are highly conserved within a given population, but which vary between different ethnic groups. Several studies have looked

at Gm and Km phenotypes in SLE with conflicting results. For example, both Schur et al. (1985) and Whittingham et al. (1983b) showed an increase in Gm heterozygosity in SLE while Stenszky et al. (1986) found no increase of Gm heterozygotes in their SLE patients. Evidence for an interactive effect between Gm and HLA types in SLE has been presented by some researchers (Whittingham et al. 1983; Stenszky et al. 1986) but not all (Schur et al. 1985). It has also been suggested that HLA-B8 and Gm homozygotes have an increased risk for renal involvement in SLE (Stenszky et al. 1987). This supports the proposal that different clinical subsets of lupus may have different genetic associations.

6.1.2 COMPLEMENT COMPONENT C3.

Complement component C3 is activated by both the classical and alternative complement pathways and therefore has a central role in complement activity. A decrease in the serum concentration of C3 is seen in active SLE, and deposition of C3 has been observed in the kidneys. C3 levels usually return to normal with remission of the disease. Little polymorphism has been observed in C3 (Alper and Propp 1968), with 99% of individuals positive for one or two alleles, C3*F and C3*S (Wyatt 1984). A few rare alleles have been reported with a frequency of less than 1%. No major functional differences have been seen between C3*F and C3*S, but Arvilommi et al. (1974) suggested that the C3*F allele may have greater affinity for the monocyte C3b receptor than C3*S. One report has shown an increased incidence of C3*F in patients with onset of SLE during childhood (McLean et al. 1980).

6.1.3 LYMPHOCYTOTOXIC ANTIBODIES.

Lymphocytotoxic antibodies against both B- and T-cells have been seen in the sera of SLE patients (Mittal et al. 1970; Stastny and Ziff 1970; Terasaki et al. 1970) as well as in people with acute viral infections (Huang et al. 1973). These LCAs may be responsible for the lymphopenia seen in some SLE patients (Winfield et al. 1975b) and have also been associated with several other clinical features (Butler et al. 1972).

6.1.4 RHEUMATOID FACTOR.

The anti-IgG antibody known as rheumatoid factor (RF) was initially seen in patients with rheumatoid arthritis, and has now also been seen in several other connective tissue diseases. RF can be induced by antigens cross reactive with IgG, polyclonal B cell activators or by aggregated IgG, for example in immune complexes (Fong et al. 1985). RF is present in 70 to 90% of rheumatoid arthritis patients, but is usually present at a much lower frequency (20%) in SLE patients. Whether these antibodies play a role in the pathogenesis of SLE or other connective tissue diseases is not known.

6.1.5 AIMS.

Gm allotyping and C3 phenotyping were performed on SLE and CTD patients to look for associations of Gm or C3 with these diseases. Lymphocytotoxic activity against B- and T-cells and rheumatoid factor activity were also examined in the SLE and CTD patients. Discriminant

analysis using the MHC class II RFLP results, C4 and C2 data, T-cell receptor RFLPs, Gm allotypes, LCAs and RF data, C3 phenotypes and other serological markers was performed to determine factors distinguishing SLE from the related CTDs. The SLE patients were also examined to look for clinical subsets that could be defined by the laboratory results.

6.2 METHODS.

6.2.1 PATIENTS AND CONTROLS.

The SLE patients and the control series have been described in Chapters 2 and 3. Patients with PSS, CREST, linear scleroderma, Sjogren's, Pm/Dm, MCTD, and UNCTD were also examined.

6.2.2 Gm TYPING.

Gm and Km typing was performed on plasma samples from SLE and CTD patients and controls by Dr. D. Propert (Royal Melbourne Institute of Technology, Melbourne).

6.2.3 C3 TYPING.

SLE and CTD patients were C3 typed as described in Chapter 2 following the method of Teisberg (1970).

6.2.4 LYMPHOCYTOTOXIC ANTIBODY SCREENING.

This was performed as described in Chapter 2. Patient sera was screened with both B- and T-cells from healthy individuals of known HLA type. The trays were read as for tissue typing, and the positive scores summed and then divided by the total number of target cells, to give a quantitative score ranging from 1.0 to 8.0.

6.2.5 RHEUMATOID FACTOR SCREENING.

Rheumatoid factor was tested for using the Rheuma-Wellcotest latex suspension (Wellcome). Agglutination reactions were scored from one to four, with four being the greatest reaction.

6.2.6 STATISTICS.

The computer statistic package SPSS^x was used in the statistical analysis, (SPSS^x User's guide 1986). Discriminant analysis (following Rao 1973) and factor analysis was performed.

6.3 RESULTS.

6.3.1 GM AND KM ALLOTYPING.

Table 6.1 gives Gm phenotyping results in SLE and CTD patients compared with the controls. The SLE patients did not differ from the controls in their distributions of Gm types, with a similar number of heterozygotes and homozygotes in the patients and controls.

TABLE 6.1 GM PHENOTYPES IN SLE AND CTD PATIENTS COMPARED WITH CONTROLS.

Gm phenotype	CON		SLE		PSS/CREST		SJOGREN'S		PM/DM		MCTD/UNCTD	
	N	%	N	%	N	%	N	%	N	%	N	%
1	5	6.8	3	3.6	4	11.8	1	14.3	0		0	
1;23	0		1	1.2	1	2.9	0		0		0	
1,2	5	6.8	2	2.4	3	8.8	0		2	14.3	3	15.8
1,2;23	0		1	1.2	0		0		0		0	
1,3;5,10	11	15.1	8	9.6	2	5.9	1	14.3	2	14.3	1	5.3
1,3;23;5,10	10	13.7	15	18.1	3	8.8	1	14.3	1	7.1	3	15.8
1,2,3;5,10	3	4.1	6	7.2	1	2.9	0		2	14.3	1	5.3
1,2,3;23;5,10	9	12.3	11	13.3	0*		1	14.3	1	7.1	1	5.3
3;5,10	5	6.8	4	4.8	2	5.9	1	14.3	0		2	10.5
3;23;5,10	24	32.9	31	37.3	17	50.0	2	28.6	6	42.9	7	36.8
1;10	0		1	1.2	1	2.9	0		0		0	
1;5,10	1	1.4	0		0		0		0		1	5.1
Number tested	73		83		34		7		14		19	

* p=.03

TABLE 6.2 KM1 ALLOTYPING IN SLE AND CTD PATIENTS AND CONTROLS.

	CON		SLE		PSS/CREST		SJOGREN'S		PM/DM		MCTD/UNCTD	
Km phenotype	N	%	N	%	N	%	N	%	N	%	N	%
Km1 positive	17	23.3	18	21.4	8	22.9	2	28.6	2	13.3	4	20.0
Km1 negative	56	76.7	66	78.6	27	77.1	5	71.4	13	86.7	16	80.0
Number tested	73		84		35		7		15		20	

TABLE 6.3 C3 PHENOTYPE AND ALLELE FREQUENCIES IN SLE AND CTD PATIENTS.

	SLE		PSS/CREST		SJOGREN'S		PM/DM		MCTD/UNCTD	
Phenotype	N	%	N	%	N	%	N	%	N	%
C3F,F	3	3.5	1	2.8	0		0		1	5.0
C3F,S	27	31.4	10	27.8	2	28.6	3	20.0	4	20.0
C3S,S	56	65.1	25	69.4	5	71.4	12	80.0	15	75.0
Number tested	86		36		7		15		20	
Allele										
C3*F	0.192		0.167		0.143		0.100		0.150	
C3*S	0.808		0.833		0.857		0.900		0.850	

The four connective tissue disease groups all showed similar distributions of Gm phenotypes compared with the controls except for the scleroderma/CREST patient group, where none of the 34 patients were positive for the 1,2,3;23;5,10 phenotype ($\chi^2_1=3.12$, $p(\text{corr})=.08$). There was a nonsignificant increase in Gm 3;23;5,10 in this disease group ($\chi^2_1=2.19$).

Table 6.2 gives the results for Km(1) typing. The proportion of patients in each disease group who are positive for Km(1) is similar to that in the control series indicating that Km(1) is not an important disease marker in SLE and the connective tissue diseases studied here.

6.3.2 COMPLEMENT COMPONENT C3.

Table 6.3 shows the C3 phenotype and allele frequencies in the SLE and CTD patients. In healthy Europeans, the frequency of the C3*S allele is about 80% (Welch and Berny 1987). All the disease groups here showed similar phenotype distributions with C3S,S the most common phenotype followed by C3F,S. The C3F,F phenotype occurred only in a small percentage of the patients. Allelic distributions are also similar between the disease groups.

6.3.3 LYMPHOCYTOTOXIC ANTIBODIES.

Lymphocytotoxins were present mainly in the lupus patients as shown in Table 6.4. LCAs against T-cells were seen in only two of the 68 CTD patients (one with CREST and one with MCTD) in contrast to 26% of the SLE patients. LCAs with B-cell reactivity were more prevalent and generally of a slightly higher titre than those against T-cells and were

TABLE 6.4 LYMPHOCYTOTOXIC ANTIBODIES IN SLE AND CTD PATIENTS.

	SLE		PSS/CREST		SJOGREN'S		PM/DM		MCTD/UNCTD	
LCA activity	N	%	N	%	N	%	N	%	N	%
T-cells										
0-2.5	53	73.6	29	96.7	6	100.0	13	100.0	18	94.7
2.6-8.0	19	26.4	1	3.8	0		0		1	5.3
Number tested	72		30		6		13		19	
B-cells										
0-2.5	52	72.2	28	93.3	6	100.0	12	92.3	17	89.5
2.6-8.0	20	27.8	2	6.7	0		1	7.7	2	10.5
Number tested	72		30		6		13		19	

TABLE 6.5 RHEUMATOID FACTOR IN SLE AND CTD PATIENTS.

	SLE		PSS/CREST		SJOGREN'S		PM/DM		MCTD/UNCTD	
RF Activity	N	%	N	%	N	%	N	%	N	%
0	48	66.7	22	75.9	0		11	91.7	8	50.0
1	14	19.4	4	13.8	1	16.7	1	8.3	2	12.5
2	5	6.9	2	6.9	1	16.7	0		2	12.5
3	3	4.2	1	3.4	1	16.7	0		1	6.3
4	2	2.8	0		3	50.0	0		3	18.8
Number tested.	72		29		6		12		16	

seen in five of 68 CTD patients (7.1%) compared with 28% of the SLE patients.

6.3.4 RHEUMATOID FACTOR.

Table 6.5 gives the rheumatoid factor results. RF was present in all the disease groups, with about 33% of lupus patients positive for RF. All the Sjogren's patients were positive for RF, with three of the six patients having the highest possible titre. About 24% of the scleroderma/CREST patients and 50% of those with MCTD or UNCTD had RF activity. RF was seen in only one of 12 patients with Pm/Dm.

6.3.5 DISCRIMINANT ANALYSIS BETWEEN SLE AND THE CTDs.

Discriminant analysis was initially carried out using the following variables: MHC class II RFLPs and DNA-DR types, C4 allotypes, C2 activity, T-cell receptor RFLPs, Gm haplotypes, C3 phenotypes, lymphocytotoxic and rheumatoid factor activity, anti-RNP, anti-SS-A, anti-SS-B and sex of the patient. These were used to find variables which might discriminate between SLE and the connective tissue diseases studied here (all the other CTDs combined). Table 6.6 gives the unstandardized canonical discriminant function coefficients of the variables which contributed to the discrimination of SLE and the CTDs. These were used to classify a patient as having either SLE or a CTD. With these variables, 54 of 71 SLE patients (76.1%) and 45 of 84 CTD patients (53.6%) were classified correctly, with 63.9% correct overall. If patients were assigned randomly to the SLE or CTD disease groups, the statistical probability of correct assignment would be 50%.

TABLE 6.6 UNSTANDARDIZED CANONICAL DISCRIMINANT FUNCTION
COEFFICIENTS WHEN ALL VARIABLES ARE USED TO
DISCRIMINATE BETWEEN SLE AND THE CTDS.

VARIABLE	COEFFICIENT
HLA-DR5	0.829
HLA-DR7	1.492
HLA-DRw10	-2.132
C4A*2	1.058
C4A*6	2.652
C4B*2	-1.142
50% C2	2.071
DR α /BglIII/4.4kb	1.435
DP β /BglIII/19kb	-0.756
TCR γ /TaqI/3.3kb	-1.524
LCAs against T-cells	0.487
Anti-RNP	-1.078
(Constant)	-3.485

TABLE 6.7 UNSTANDARDIZED CANONICAL DISCRIMINANT FUNCTION
COEFFICIENTS OF VARIABLES USED TO DISCRIMINATE
BETWEEN SLE AND THE CTDS.

VARIABLE	COEFFICIENT
HLA-DR3	0.966
C4B*2	-0.857
TCR β /BglIII/9.2kb	0.577
LCAs against B cells	0.470
RF	-0.492
(Constant)	-1.381

When results for a chosen variable were not available for a particular patient, the patient was excluded from the initial analysis of the significance of the variables (discriminant step), and the computer analysis was carried out on a smaller number of patients than those used in the final classification step. This means that the number of patients can vary slightly depending on the variables used. Therefore, patients for whom there were no LCA and RF results (27 patients) were excluded from the next analysis, as were some of the variables which were not useful in discriminating between SLE and the other CTDs in the initial analysis.

In subsequent analyses, variables which did not contribute to the discrimination were removed. When the variables DR3, C4B*2, RF, lymphocytotoxins against B-cells, and the TCR β /BglIII/9.2kb RFLP were used, 45 of 60 or 75% of the SLE patients and 53 of 68 (77.9%) of the CTD patients were classified correctly, with 76.6% correct overall. The unstandardized canonical discriminant function coefficients are shown in Table 6.7. The discriminant analysis could not be improved beyond this point when other variables were included.

A correlation matrix of the variables HLA-B8, DR3, C4B*2, LCAs against B- and T-cells, RF and the TCR β /BglIII/9.2kb RFLP is shown in Table 6.8. As expected, HLA-B8 and DR3 showed a high level of correlation as do the LCAs against B- and T-cells. However, replacing DR3 with the joint occurrence of B8 and DR3 in the discriminant analysis did not improve the classification of the patients, with 92 (71.9%) of the patients being correctly classified when B8,DR3 was used and 98 (76.6%) correctly classified when DR3 was used. Similarly, T-cell LCAs were not as informative as B-cell LCAs in the discriminant analysis, with 91 (71.1%) of the patients grouped correctly when T-cell LCAs were

TABLE 6.8 CORRELATION MATRIX OF VARIABLES DISCRIMINATING BETWEEN SLE AND THE CTDS.

DISEASE	<u>HLA-DR3</u>	<u>HLA-B8</u>	<u>LCAT</u>	<u>LCAB</u>	<u>RF</u>	<u>TCRβ/9.2</u>	<u>C4B*2</u>
DISEASE	1.000						
HLA-DR3	-.188	1.000					
HLA-B8	-.104	.762	1.000				
LCAT	-.255	-.094	-.036	1.000			
LCAB	-.345	-.030	.017	.762			
RF	.160	.187	.194	.189	1.000		
TCRβ/9.2	-.064	.083	.099	.144	.059	1.000	
C4B*2	.142	-.126	-.212	.128	.051	.087	1.000

DISEASE= SLE versus CTDS

LCAT=lymphocytotoxins against T-cells

LCAB=lymphocytotoxins against B-cells

TCRβ/9.2= TCRβ/BgIII/9.2kb RFLP

included compared with 98 (76.6%) correctly grouped when B-cell LCAs were used.

6.3.6 ANALYSIS OF CLINICAL SUBSETS OF SLE.

The SLE patients were divided into two groups depending on the presence or absence of particular ARA classification criteria. The number of patients with or without malar rash, discoid rash, oral ulcers, arthritis, pericarditis, proteinuria, renal disorders, seizures, psychosis, haemolytic anaemia, leukopenia, lymphopenia, thrombocytopenia, positive LE cell prep, anti-Sm, false positive test for syphilis and antinuclear antibodies was less than ten in the initial analysis when all variables were included, so discriminant analysis was not performed on these classification criteria.

The classification criteria tested were presence or absence of photosensitivity, pleuritis and anti-DNA antibodies. The discriminant analysis was initially carried out with all available variables and those which were not significant were then removed from the analysis, resulting in a smaller number of variables which could be used to define these three classification criteria.

6.3.6.1 PHOTSENSITIVITY.

Presence or absence of photosensitivity was best defined by the following variables: HLA-DR4, C4A*2, anti-SS-A, and the DP β /BgIII/19kb RFLP. (Unstandardized canonical discriminant function coefficients are given in Table 6.9). In this analysis, 34 of 44 (77.3%) of the photosensitive patients were grouped correctly and 19 of 28 (67.9%) of

TABLE 6.9 UNSTANDARDIZED CANONICAL DISCRIMINANT FUNCTION
COEFFICIENTS OF VARIABLES USED TO DISCRIMINATE
SLE PATIENTS WITH PHOTSENSITIVITY.

VARIABLE	COEFFICIENT
HLA-DR4	0.809
C4A*2	-1.074
DP β /BgIII/19kb	-1.501
Anti-SS-A	1.731
(Constant)	0.477

TABLE 6.10 UNSTANDARDIZED CANONICAL DISCRIMINANT FUNCTION
COEFFICIENTS OF VARIABLES USED TO DISCRIMINATE
SLE PATIENTS WITH PLEURITIS.

VARIABLE	COEFFICIENT
HLA-DR1	-2.290
HLA-DRw8	-2.411
DR α /BgIII/3.9kb	1.071
DP β /EcoRV/12kb	1.652
DP β /EcoRV/8kb	2.240
TCR β /BgIII/9.2kb	-1.880
(Constant)	-0.175

TABLE 6.11 UNSTANDARDIZED CANONICAL DISCRIMINANT FUNCTION
COEFFICIENTS OF VARIABLES USED TO DISCRIMINATE
SLE PATIENTS WITH ANTI-DNA ANTIBODIES.

VARIABLE	COEFFICIENT
HLA-DRw8	2.902
HLA-DRw14	2.528
DR α /BgIII/3.9kb	-1.194
TCR α /EcoRV/1.7kb	-1.456
LCAs against B-cells	0.486
Anti-SS-B	-1.319
Sex	2.050
(Constant)	-3.810

those not photosensitive were correctly classified, with 73.6% correct overall. The four discriminating variables were then examined in relation to the presence or absence of photosensitivity. Anti-SS-A was significantly increased in patients with photosensitivity, ($\chi^2_1=4.0$, $p(\text{corr})=.04$), with 11.8% of the photosensitive negative patients being anti-SS-A positive, and 34.1% of the patients who were photosensitive being anti-SS-A positive. Of the remaining three variables, DR4 was increased and the DP β /BglIII/19kb RFLP was decreased in the photosensitive positive patients, but neither was statistically significant. The C4A*2 allele may have been included in the discriminant function because of the small number of individuals carrying this allele (four who were photosensitive, two who were not).

6.3.6.2 PLEURITIS.

The variables HLA-DR1 and -DR8, DP β /EcoRV/12kb and 8kb RFLPs, TCR β /BglIII/9.2kb and DR α /BglIII/3.9kb RFLPs and were the most useful in discriminating patients with pleuritis. With these variables, 20 of 29 (69.0%) of patients with pleuritis were classified correctly, while 37 of 43 (86.0%) of patients without pleuritis were correctly grouped, with 79.2% correct overall. Table 6.10 gives the unstandardized canonical discriminant function coefficients. The discriminating variables in this analysis were correlated with the presence or absence of pleuritis, and the DP β /EcoRV/12kb RFLP was significantly decreased in patients with pleuritis, being present in only 41.9% of pleuritis positive patients compared with 73.9% of pleuritis negative patients ($\chi^2_1=6.7$, $p(\text{corr})=.01$). The DP β /EcoRV/12kb fragment correlates with the DP β /BglIII/19kb RFLP, and this fragment was similarly decreased in the

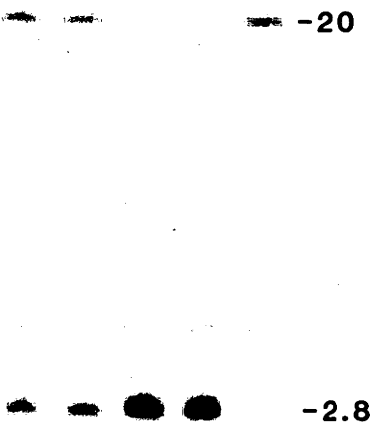
FIGURE 6.1 a. DP β hybridization of EcoRV digested DNA.

b. DP β hybridization of BglII digested DNA.

a. DP β /EcoRV



b. DP β /BglII



patients with pleuritis, but was not a useful variable in the discriminant analysis when the 12kb EcoRV fragment was included (DP β RFLPs are shown in Figure 6.1). HLA-DR1, the DR α /BglIII/3.9kb and the TCR β /BglIII/9.2kb RFLPs were all decreased in patients with pleuritis, but none was statistically significant. HLA-DRw8 was probably included in the discriminant function because of the low frequency of this allele, with only one patient HLA-DR8 positive (pleuritis positive).

6.3.6.3 ANTI-DNA ANTIBODIES.

Patients with anti-DNA antibodies were best defined by the variables HLA-DRw8, HLA-DRw14, LCAs against B-cells, DR α /BglIII/3.9kb and TCR α /EcoRV/1.7kb RFLPs, anti-SS-B, and sex. In this analysis, 49 of 55 (89.1%) of patients with anti-DNA antibodies and 10 of 17 (58.8%) of patients without anti-DNA antibodies were classified correctly, with 81.9% correct overall. (The unstandardized canonical discriminant function coefficients are given in Table 6.11). Only 5% of the patients without anti-DNA antibodies were males, compared with 18.5% of patients with anti-DNA antibodies being male (not statistically significant). HLA-DRw8 and DRw14 were present at a low frequency (one DRw8 positive and four DRw14 positive patients, all with anti-DNA antibodies), which probably accounts for their inclusion in the discriminant analysis. Alternatively, in combination with the other variables in the discriminant function, HLA-DRw8 and DRw14 may be important discriminating variables. The TCR α /EcoRV/1.7kb and DR α /BglIII/3.9kb RFLPs did not show significantly different distributions in the anti-DNA positive patients and the anti-SS-B antibodies were present in a similar number of patients with and without anti-DNA

antibodies. It is possible that a combination of these variables may be important in patients with anti-DNA antibodies.

6.4 DISCUSSION.

6.4.1 DISCRIMINATION OF SLE FROM CTDs.

Lymphocytotoxic activity against B-cells was one of the most important variables in the discriminant analysis between SLE and the CTDs. Although only 28% of the SLE patients had LCAs against B-cells, very few (7%) of the CTD patients had these antibodies. A much higher incidence of LCAs in lupus has been observed in other studies, with up to 80% of patients reported to be positive for LCAs (Malave et al. 1976; Mittal et al. 1970; Persselin et al. 1977). However, a study of IDDM patients (Serjeantson et al. 1981), has shown that the level of LCAs decreases dramatically from the time of disease onset. It is not known whether a similar phenomenon occurs in SLE, because SLE is not always diagnosed at "onset" because of the diversity of clinical features which make diagnosis difficult. A patient may have less than the required number of ARA criteria for some time before SLE is officially diagnosed, and by that time the level of LCAs may have dropped considerably. This study included patients who had SLE for some time, which could account for the lower level of LCAs observed in this series. Also, lymphocytotoxic antibodies have been reported to increase with disease activity in lupus (Butler et al. 1972; Winfield et al. 1975b), and the patients studied here did not necessarily have active disease at the time of blood collection. LCAs may well be an important pathogenic factor in SLE as indicated by the discriminant analysis here.

DR3 was also included in the discriminant function, which is consistent with the results presented in Chapter 3 where DR3 was significantly increased in the SLE patients. Other class II RFLPs do not appear to contribute to the discriminant analysis between SLE and the CTDs. Surprisingly perhaps, when the joint occurrence of B8 and DR3 was used in the discriminant analysis instead of DR3 alone, (because B8 and DR3 are in linkage disequilibrium with the C4A null allele), a slight drop was seen in the number of patients correctly classified. This is because not all C4A null alleles are necessarily on the B8.DR3 haplotype, (Batchelor et al. 1987), and because patients positive for both B8 and DR3 did not necessarily carry these alleles on the same chromosome, despite the high level of linkage disequilibrium between them. Complete C4A deficiency was not a significant variable in the discriminant analysis, because of the small number (6) of individuals with complete C4A deficiency. As described in Chapter 4, DR3 is a "marker" for the C4A null allele and its contribution to SLE is secondary to partial and complete C4A deficiency. Partial C4A deficiency was not included in the discriminant analysis because of the difficulty in assigning some null alleles.

Rheumatoid factor and C4B*2 also contributed to the discriminant analysis. RF is present in many connective tissue diseases including rheumatoid arthritis, Sjogren's syndrome, MCTD and UNCTD. RF selected for the CTD patients rather than the SLE patients as shown by the negative value in function 1. C4B*2 which correlates negatively with C4A*Q0, had a higher frequency in the CTDs than in SLE and contributed to the discriminant analysis. This enforces once again the importance of complete or partial C4A deficiency in SLE. The positive contribution of the TCR β /BgIII/9.2kb RFLP to the selection of the SLE patients

reflects the significant decrease of this fragment in the CTD patients (Chapter 5).

No Gm phenotypes contributed significantly to the discriminant analysis between SLE and the CTDs, nor were Gm phenotype distributions different in SLE compared with the controls. This is in contrast to other studies where Gm heterozygosity was increased (Whittingham et al. 1983; Schur et al. 1985) and where interaction between Gm and HLA phenotypes was seen (Whittingham et al. 1983; Stenszky et al. 1986). Stenszky et al. (1986) have also reported a role for Gm and HLA-B8 in patients with renal involvement, but this was not seen in another study (Schur et al. 1985). The variables here could not successfully discriminate between patients with and without renal involvement, so the role of Gm could not be fully investigated.

C3 does not appear to be important in the SLE patients studied here, either in the discriminant analysis or the phenotypic distributions, which is in agreement with another recent study (Welch and Berny 1987). Of the clinical features examined by discriminant analysis, (photosensitivity, pleuritis, and anti-DNA antibodies), C3 was not a significant variable.

6.4.2 CLINICAL SUBSETS OF SLE.

The three classification criteria analysed by discriminant analysis could be partly defined by some of the genetic variables. For example, pleuritis patients showed a significant decrease in the DP β /EcoRV/12kb fragment which correlates with DPw1, w3, w5, and w6 (Hyldig-Nielsen et al. 1987). The alternate fragment (8kb) was not increased suggesting that there was an increase of DPw2 and DPw4 homozygotes in the pleuritis

positive patients. This would need to be further examined to see if there was a role for DP in pleuritis. Of the clinical variables, anti-SS-A antibodies were strongly associated with photosensitivity which is in accord with earlier clinical studies associating anti-SS-A antibodies with skin lesions (Gilliam and Sonthheimer 1982). Exposure to sunlight exacerbates most skin problems in lupus.

The three classification criteria examined did have different combinations of variables which were important in the discriminant analysis, supporting the hypothesis that different genetic variables are responsible for various clinical features. Not all the patients were correctly classified with respect to the presence or absence of particular classification criteria, but patients are positive for at least four of the ARA criteria and therefore could be expected to have four different genetic factors involved, which makes it harder to select for one primary genetic variable being associated with one clinical feature.

6.4.3 GM AND C3 IN THE CTD PATIENTS.

Only the PSS/CREST patients showed a difference in Gm phenotype distributions, with none of the patients carrying the 1,2,3;23;5,10 phenotype. However, none of the Gm specificities were increased or decreased, nor was the homozygote to heterozygote ratio different in this patient group, so the absence of this phenotype may be a function of small sample size. A larger patient group will need to be examined to see if this is indeed a significant finding. The other CTDs examined have too few patients to make any definite conclusions about the role of Gm in these diseases. Complement component C3 did not appear to play an

important role in the connective tissue diseases studied here, although patient numbers in some of the disease groups are low.

6.5 CONCLUSION.

Discriminant analysis between SLE and the CTD patients has shown that lymphocytotoxic activity against B cells is an important differentiating variable and may play a more important role in the pathogenesis of SLE than was previously thought. DR3 was the only MHC class II antigen associated with SLE which supports the findings of Chapter 3 and reflects linkage disequilibrium with the C4A null allele. Gm and C3 do not appear to be associated with SLE or clinical subsets of SLE. Some genetic heterogeneity in clinical subsets of SLE was observed, but no distinguishing genetic factor was found.

CHAPTER SEVEN

GENERAL DISCUSSION: MECHANISMS OF DISEASE IN SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus is one of many diseases that has shown associations with the genes of the major histocompatibility complex. Disease associations with MHC genes are rarely absolute, with the notable exception of narcolepsy, (Juji et al. 1985; Langdon et al. 1984), and this is often attributed to linkage disequilibrium between genes in the MHC, which can make it difficult to distinguish between a disease susceptibility gene and a gene merely acting as a "marker" for the actual disease promoting locus. However, there is the possibility of more than one gene within the MHC contributing to a disease. In addition, an MHC gene and genes outside the MHC (for example the T-cell receptor or immunoglobulin genes) may also influence inherited disposition to a disease. For example, in IDDM it has been estimated that MHC genes contribute, at the most, about 60% of the genetic susceptibility (Rotter and Landlaw 1984). It is also likely that environmental agents play a role in several MHC associated diseases including systemic lupus erythematosus as suggested by a lack of concordance in family and twin studies (Arnett et al. 1984).

The mechanisms involved in the MHC class I and II gene disease associations are not clear, although several mechanisms have been proposed, as recently reviewed by Batchelor and McMichael (1987). The MHC class I and II HLA genes encode the proteins which are involved in the presentation of antigen on the cell surface, resulting in recognition of foreign antigen by the T-cell receptor, and a full immune response. There are several ways in which the HLA proteins could be involved in susceptibility to a disease, including (1) HLA antigens as virus receptors (2) molecular mimicry (3) control of immune responsiveness.

The HLA antigens could act as receptors for viral pathogens, thus facilitating viral entry into a cell. Experiments testing this hypothesis have given conflicting results. For example, one study showed that the HLA-A and -B antigens in man and the equivalent H-2K and -2D antigens in mouse were acting as receptors for Semliki Forest virus (Helenius et al. 1978). However, it has been reported that Semliki Forest virus can infect and replicate in murine cell lines which do not express H-2 antigens (Oldstone et al. 1980). In contrast to this, another report by Oldstone et al. (1975) indicated that this same virus could not replicate in Daudi cells which do not express HLA antigens. A recent study has shown that human cytomegalovirus (HCMV), which usually affects immunosuppressed patients, has a gene which is very similar to the HLA class I genes (Beck and Barrell 1988). HCMV has been shown to bind to the β 2-microglobulin protein of the class I antigens (Grundy et al. 1987a), raising the possibility that binding to the β 2-microglobulin may be an infection mechanism of the virus (Grundy et al. 1987b). It has yet to be shown if the HCMV uses this newly discovered HLA-like protein to bind to the HLA antigens. In the case of SLE, there is not a 100% concordance between the disease and a particular HLA antigen, nor are there any HLA antigens seen in SLE which are not seen in a healthy population, which argues against an HLA antigen acting as receptor for a viral pathogen. However, it should be noted that non-antigenic molecular variation occurs in class I (Strominger 1987) and class II (Todd et al. 1987) antigens. HLA-DR3 was increased in the Caucasoid lupus patients studied here, but is also present at a significant level in the general population, suggesting that other factors are more important. DNA sequence data have not revealed heterogeneity in the DR β 1, DQ α or DQ β genes in DR3 positive individuals,

(Boss and Strominger 1984; Gorski and Mach 1986; Bell et al. 1987; Todd et al. 1987), although DR3 is associated with two different DR β 3 genes (Gorski and Mach 1986; Tiercy et al. 1988). The present study has shown an increase in DR3, particularly on the B8.DR3 haplotype which can be differentiated from B18.DR3 by RFLP analysis (Chapter 3). However, the B8.DR3 haplotype is common in healthy Caucasoid individuals, which does not support a particular DR3 variant acting as a viral receptor in SLE.

Another mechanism put forward to explain HLA associations with disease is that of molecular mimicry. The HLA proteins could be serologically cross reactive with infectious organisms resulting in a failure to mount an immune response, or, a reaction against these pathogens could result in autoantibodies. Evidence for mimicry comes from HLA-B27 and diseases with which it has been associated, for example ankylosing spondylitis, (AS), (reviewed by Tiwari and Terasaki 1985). Klebsiella pneumoniae has been isolated from patients with AS at a higher rate during active disease (Ebringer et al. 1977), and B27 has been shown to cross react with antibodies to K.pneumoniae, while antibodies to B27 react with K.pneumoniae (Ebringer et al. 1976). However, another study did not confirm these results (Archer et al. 1981). B27 is also increased in frequency in patients with Reiter's disease and reactive arthropathy, which develop after large bowel or urinary tract infections with Shigella, Salmonella, Yersinia or Chlamydia or other bacteria. Molecular mimicry may be involved as the B27 association is very high, particularly with AS, and no association with the class II DR antigens has been seen in these diseases, although an immune response should not develop against self-like antigens, and an anti-B27 antibody has not yet been found in AS patients. It is possible that those antigenic sites with self-like epitopes on a pathogenic agent

could cause an increase in T-suppressor cell activity, therefore decreasing T-helper cell activity against other more immunogenic sites on a pathogenic agent, and letting an infectious agent persist longer (Batchelor and McMichael 1987).

A third hypothesis put forward is that the HLA genes are "immune response" genes which influence an individuals response to foreign antigens. Immune response genes have been mapped in the mouse, (reviewed by Benacerraf and Germain 1978), and are believed to be equivalent to the HLA-D/DR genes in man (Sasazuki et al. 1983). Support for this comes from diseases that were initially associated with the HLA-B antigens later showing stronger associations with the DR genes. Also, HLA-B associations with a particular disease can differ between various ethnic groups which could be explained by different linkage disequilibrium relationships with the same immune response gene. One study (Melief et al. 1986) has shown that a mouse strain with three changes in the H-2K gene does not respond to Sendai virus and dies while the normal mouse strain does respond immunologically, suggesting that the H-2 antigen determines if there will be a response to a foreign antigen. Studies of man have shown that isolated populations which have been subjected to a virulent disease, can have different HLA profiles compared to the population from which they originated. For example, descendants of the Dutch people in Surinan who had to survive typhoid and yellow fever epidemics after emigration from Holland, have different HLA antigen frequencies to those who remained in Holland (De Vries et al. 1979). The HLA antigens B8 and/or DR3 which are increased in lupus studies have been associated with impaired responsiveness in vitro to phytohaemagglutinin, concanavalin A and α -helix haemocyanin, (Amer et

al. 1986; McCombs and Michalski 1982; Kallenberg et al. 1981), which is suggestive of immune response involvement by the HLA antigens.

HLA antigens are not necessarily the only MHC genes involved in disease susceptibility. Congenital adrenal hyperplasia (CAH) is associated with certain HLA haplotypes which is clearly explained by linkage disequilibrium with the class III 21-OH genes one of which, (21-OHB), is partly deleted in patients with CAH (White et al. 1984). The 21-OHA gene is not believed to be active in the general population (White et al. 1986). Without the 21-hydroxylase enzyme, there is a lack of cortisol, a subsequent increase in adrenocorticotrophic hormone, and therefore a build-up in the precursors to cortisol. An excess of androgens results in CAH, in both the simple virilization and salt wasting forms, both of which show different HLA haplotype associations.

The complement genes could also be involved in susceptibility to certain diseases, in particular to systemic lupus erythematosus. Early studies showed associations with the HLA class I antigens B5 and B8, and as serology developed, with the class II antigens DR2 and DR3 (Tiwari and Terasaki 1985). However, it is now becoming clear from several studies that SLE shows stronger associations with the class III loci than with the HLA genes, in particular with deficiencies of complement components C2 and C4. Several studies including this one have now focussed attention specifically on the deficiency of C4A (Christiansen et al. 1983; Howard et al. 1986; Kemp et al. 1987), and this is seen not only in Caucasoids, but also several other non-Caucasoid populations including American blacks, Japanese and Chinese, where different linkage relationships exist. The common locus between different ethnic groups is more likely to be the one most important in the disease. SLE is characterized by immune complexes which are believed to cause much of

the tissue damage seen in lupus, so a complement deficiency is very likely to be pathogenic as it would allow a build-up of immune complexes, facilitating immune complex precipitation. In addition to the important role of C4A in binding to amino nucleophiles on immune complexes, (discussed in Chapter 4), the classical complement pathway, of which C4 is a part, has been shown to be more important than the alternative pathway in the prevention of immune precipitation (Webb and Whaley 1986; Schifferli et al. 1986a). This indicates the importance of a fully functional classical pathway to stop the initial precipitation of immune complexes which would cause tissue damage. However, not all the lupus patients studied here have C4A deficiencies, suggesting genetic heterogeneity in SLE.

C4B deficiency was not implicated in this study, although other researchers have reported an increase of C4B null alleles in lupus patients (Fielder et al. 1983; Reveille et al. 1985), particularly in DR3 negative patients (Batchelor et al. 1987). It could be that deficiency of C4B does play a role in lupus, but that it is not as damaging as C4A deficiency (as indicated by the ethnic study and logistic analysis results in Chapter 4). In this study, neither complete or partial C4B deficiency increased susceptibility to lupus, even when HLA-DR3 status was statistically controlled in the logistic regression. The differing results may be due to clinical heterogeneity between patient study populations. However, the study of the DR3 negative patients does support the hypothesis that complement deficiency is a pathogenic factor in lupus.

It is also possible that different complement deficiencies may be present in different clinical subgroups of SLE. For example, it has been reported that the clinical presentation in patients with lupus

associated with C2 deficiency does differ from classical lupus. This includes an increase in discoid lesions, a decrease in the incidence of renal disease, lower titres or no ANAs and anti-DNA antibodies, and less Ig and complement deposits in skin lesions (reviewed by Agnello 1986). While there were decreased levels of C2 in many of the lupus patients in this series (Chapter 4), this did not appear to be due to inherited C2 deficiency. Similarly, another study in Australia (Christiansen et al. 1983) reported no increase of inherited C2 deficiency in their lupus patients. However, studies elsewhere have shown C2 deficiency to be increased in SLE (Glass 1976; Rynes 1982). These conflicting results could once again be partly explained by clinical heterogeneity in the patient series. However, because C4 reacts directly with immune complexes, deficiency of C4 may be more critical in SLE than that of C2.

Another indication that the complement system is important in lupus comes from studies of the CR1 receptor (reviewed by Atkinson 1986; Walport and Lachmann 1983). CR1 binds to either C3b or C4b, which in turn are bound to immune complexes, thus facilitating removal of immune complexes from the circulation. CR1 is found on B cells, granulocytes, macrophages and monocytes, but the majority are found on erythrocytes due to the large number of red blood cells in the blood. This means that immune complexes can be quickly transported to the liver where they are removed. Studies of CR1 have shown a decrease in the number of receptors on RBC in SLE patients (Iida et al. 1982; Wilson et al. 1984) and also reduced adherence of immune complexes to CR1 (Miyawaka et al. 1981). At this time it is not clear if these differences are inherited (Wilson et al. 1982), or acquired as a result of lupus (Iida et al. 1982; Inada et al. 1982; Kazatchkine et al. 1982; Uko et al. 1985), although the evidence tends to support an acquired defect. However, it

is clear that a defect in this receptor, (either by reduced numbers or reduced binding capacity), would result in slower clearance of immune complexes from the circulation, so there would be more chance of immune complex precipitation and deposition and therefore tissue damage. The C4, C2, and CR1 studies all point to an important role for the complement system in SLE. However, these cannot be the only genes involved in lupus as C4 and C2 deficiencies, and lower levels of CR1 are also seen in healthy individuals.

Interest is now focussing on the region between the class I and class III genes of the MHC, where the tumor necrosis factor genes have recently been mapped (Dunham et al. 1987; Carroll et al. 1987). A recent report (Jacob and McDevitt 1988) has shown that NZW mice have a RFLP in the TNF- α gene which correlates with decreased levels of the TNF- α protein. When replacement therapy with a recombinant TNF- α protein was performed, there was a delay in the onset of lupus nephritis and a longer survival rate in the (NZB x NZW)F1 mice. Untreated F1 mice had a 95% mortality rate in 12 months compared to 30% in the same time in the TNF- α treated mice. Now that the human TNF genes have been cloned, studies of this gene should be undertaken in SLE patients to see if a similar role for TNF may be found in man also.

There is also the possibility of new genes being found in the area of the MHC between the class I and III genes, which could play a part in diseases. Pulsed field gel electrophoresis has shown that the A1.B8.DR3 haplotype carries a large deletion of about 40kb between the TNF and HLA-B genes (Tokunaga et al. 1988) which is of interest in studies of SLE, as many lupus patients have this HLA haplotype. Ethnic studies may be useful in studying this region as shown for the complement genes in

this study, to see if there is a common factor in this region which is associated with SLE.

Studies of murine lupus models indicate that genes outside the MHC are also involved in the pathogenesis of SLE, for example, the lpr gene in MRL mice and Y-chromosome linked genes in BXSB mice. Some studies have also suggested that the T-cell receptor genes play a role, but these have been discrepant (Yanagi et al. 1986; Kotzin et al. 1987). No association was seen with the TCR RFLPs in this study (Chapter 5). If it was possible to examine a more restricted T cell population, as was done by Savill et al. (1987) when looking at synovial fluid from patients with rheumatoid arthritis, unusual TCR gene rearrangements may be seen. However, because of the systemic, diffuse nature of SLE it will be difficult to find a restricted population. A probe specific for the variable region of the TCR genes might also be useful to see if there is restriction in the V genes that are used by SLE patients.

This study showed no change in Gm phenotypes in SLE patients compared with healthy controls, in contrast to several other studies (Whittingham et al. 1983; Schur et al. 1985; Stenszky et al. 1986). This may be explained by clinical or ethnic heterogeneity in the different studies. Use of DNA probes for the immunoglobulin genes would be interesting and may reveal more heterogeneity than is seen at the protein level as has been shown for the HLA genes. For instance, heterozygotes and homozygotes for the Gm23 specificity can be differentiated by RFLP studies (Jazwinska et al. 1988) which is not possible by serology, except in family studies.

It is still not clear what causes the vast array of autoantibodies which are seen in lupus patients. A possible mechanism is that infection by a virus in susceptible individuals results in death and

destruction of cells, releasing DNA, nucleoproteins and other cellular contents into the tissues and circulation. Because these are not normally seen by the immune system, T-and B-cells may not have been selected for nonreactivity with these antigens. Therefore, autoantibodies are produced, and individuals with deficiencies in the complement system, particularly C4A, would not properly clear the resulting immune complexes, which would precipitate, causing tissue damage. This in turn would result in more cellular antigens being released and another cycle of immune complex damage. However, this is only one possible mechanism and does not explain all the features of lupus, such as the higher incidence of SLE in women.

This study has shown the value of genetic analyses in understanding underlying disease mechanisms in an autoimmune disease for which the aetiology is unknown. This should ultimately lead to improved patient management and treatment. The earlier reports of HLA-DR associations with SLE focussed attention on the immune system and possible immunological dysfunction in lupus. However, this study, together with other recent publications, suggests a primary role for the complement pathway in contributing significantly to the the pathogenesis of systemic lupus erythematosus.

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